University of Alberta

Cyclosporine and Tacrolimus: Mechanisms of Action

by

Lina Kung

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Medical Microbiology and Immunology

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Aug 2, 2001

"It does not matter how slowly you go so long as you do not stop."

Confucius
551 BC - 479 BC

University of Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Cyclosporine and Tacrolimus: Mechanisms of Action" submitted by Lina Kung in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Although cyclosporine (CsA) and tacrolimus (FK) have been used as immunosuppressive agents for two decades, their mechanisms of action *in vivo* are not completely understood. For example, these two drugs have similar effects but at different concentrations. This thesis addresses the molecular bases for the quantitative differences between CsA and FK as well as the bases for their tissue-specific effects.

The data presented here support the model that differential inhibition of calcineurin (CN) is a major reason for the quantitative difference between CsA and FK. The differences in concentrations (IC50's) required for 50% inhibition of CN, dephosphorylation of nuclear factor of activated T cells (NFATC), and interferon (IFN)-γ secretion correlated with drug dose, therapeutic blood concentrations, as well as with the effects of these two drugs on *in vitro* immune responses. In patients, CN activity was also comparably inhibited at FK doses 30-fold lower than CsA. The IC50 for *in vitro* CN inhibition by FK in whole human blood was also ~20-fold lower and consistent with therapeutic trough levels.

These studies led to two surprising observations. First, although both drugs completely inhibited NFATC2 dephosphorylation and IFN-γ secretion, neither completely inhibited CN activity assayed.

Furthermore, FK inhibited less than CsA despite its greater molar potency. CN inhibition by CsA and FK could be made complete with the addition of the cyclophilin A (CyPA) or FK506 binding protein 12 (FKBP12), respectively. This suggested that immunophilins were limiting for CN inhibition. Among mouse tissues, maximum CN inhibition by CsA and FK also differed. FKBPs were generally more limiting than CyPs and this observation correlated with the lower protein expression of FKBP12 vs. CyPA in mouse tissues. The second surprising observation was that the *in vitro* IC50s for CN inhibition by FK in tissue homogenates were in the same range as those for CsA. The discrepancy between these and earlier results was due to cell disruption. The IC50 for FK but not CsA was lower if intact cells were treated rather than cell lysates. Finally, I showed that the mechanism of CsA *in vitro* and *in vivo* is independent of transforming growth factor (TGF)-β1.

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List of Abbreviations

95% CI	95% confidence interval
AID	autoinhibitory domain
AKAP79	a 79-kD protein kinase A anchoring protein
APC	antigen presenting cell
CaM	calmodulin
CN	calcineurin
CNA	calcineurin A subunit
CNB	calcineurin B subunit
CsA	cyclosporine
CyP	cyclophilin
DBD	DNA binding domain
FK	tacrolimus or FK506
FKBP	FK506 binding protein
IC50	concentration inhibiting 50% activity
IL-	interleukin-
IFN-γ	interferon-γ
iono	ionomycin
IP_3	inositol triphosphate
LAP	latency associated peptide
LTBP	latent TGF-β binding protein

MEF-2	myocyte enhancer factor –2
NFATC	nuclear factor of activated T cells
NFκB	nuclear factor κ B
NHR	NFAT homology region
NLS	nuclear localisation sequence
NOS	nitric oxide synthase
PBL	peripheral blood leukocytes
PGP	P-glycoprotein
SCS	spleen cell suspension
TβR	TGF-β receptor
TGF-β	transforming growth factor - β

Chapter One

Background

I. Milestones in Transplantation

Transplantation is the process of transferring cells, tissues, or organs from one location to another. An autograft is a transfer within the same individual. Isografts and allografts are between genetically identical and non-identical individuals, respectively. Xenografts cross the species barrier. The first documented experiment in organ transplantation was in 1908 when Alexis Carrel interchanged the kidneys of cats. Although none of the transplanted kidneys lasted more than 25 days, Carrel successfully demonstrated that a transplanted organ could function normally in the recipient. Little and Tyzzer described the first genetic relationships in transplantation and these findings were the basis for the three laws of transplantation: (1) isografts accept, (2) allografts fail, (3) acceptance is governed by multiple co-dominant Mendelian factors (1). The first human-to-human kidney transplant was attempted in the 1930s by Dr. Y. Voronoy. The attempt however was unsuccessful because the cadaver kidney was transplanted to a recipient with a different blood type.

The first major advancement in transplantation is the discovery of the human ABO blood groups by Karl Landsteiner, who subsequently received the Nobel Prize in Medicine in 1930 for this work. Matching donor and recipient ABO antigens is crucial not only for the success of blood transfusions but also solid organ

transplantation. Blood vessels of the graft also express these blood group antigens and are thus also subject to attack if recognized as foreign by the recipient. ABO matching however is not enough to prevent graft rejection. In 1936, Peter Gorer showed that there were separate blood groups in serum and that the histocompatibility antigen 2 (H2) was associated with strong tumor rejection (2).

In the 1940s, observations by Sir Peter Brian Medawar shed some light on the basis for graft rejection and became the next major development in transplantation. As predicted by the laws of transplantation, Medawar observed that skin grafted within the same patient (autograft) was accepted while skin grafted from relatives (allograft) was rejected (3). Medawar also found that allografts attempted a second time between the same donor and recipient were rejected even faster and with more fervor. In animal experiments, he demonstrated that prior sensitization of the recipient with donor cells resulted in a more intense rejection of the allograft (4). Medawar subsequently put forth the theory that the rejection of a transplant is based on immunologic factors.

In the 1950s, the role of the immune system in transplant rejection was further clarified. Rupert Billingham, Leslie Brent, and Peter Medawar reported the ability to "adoptively" transfer immunity to skin grafts and other tissues in the mouse through lymphoid cells (5).

Other researchers demonstrated that lymphocytes, even in the absence of antibody, could directly attack the transplant. In 1951, George Snell discovered the K and D loci of H2 (6). The following year, Jean Dausset described the histocompatibility complex genes in humans and this was the basis for tissue typing using leukocytes (7). Jean Dausset later shared the 1980 Nobel Prize in Medicine with Baruj Benacerraf and George D. Snell for the discovery of the major histocompatibility complex (MHC).

In 1954, the first successful kidney transplant was between identical twins and performed by Dr. Joseph E. Murray, Dr. John Hartwell Harrison, and Dr. John P. Merrill at the Brigham & Women's Hospital in Boston (8). Dr. Murray later received the Nobel Prize in Medicine for this achievement. Despite this and subsequent successes in monozygotic twins, the immunologic hurdle in transplanting between genetically non-identical individuals remained; allografts were still attacked and destroyed by the immune system of the recipient. The mechanisms of graft rejection are discussed below.

II. Mechanisms of Graft Rejection

Graft rejection can be separated into three general categories based on its time course: hyperacute rejection, acute rejection, and chronic rejection. Hyperacute rejection occurs within the first 24 hours

and is mediated by host serum antibodies specific for the antigens of the graft (MHC, ABO blood antigens) (9-11). The antigen-antibody complexes trigger the complement system, resulting in the infiltration of polymorphs and an inflammatory reaction that destroys the endothelium. Acute rejection begins around the first week following transplantation when the first signs of infiltration, inflammation, injury, and death are seen (12). Effector mechanisms include delayed type hypersensitivity, cytotoxic cells (direct lysis), or antibody. Antibody-dependent mechanisms include complement, antibody dependent cellular cytotoxicity, or Fc receptor mediated activation of macrophages. Chronic rejection occurs months to years after transplantation and is characterized by intimal thickening in small arteries, progressive parenchymal atrophy, and interstitial fibrosis (13). Initially successful, the transplant may gradually lose its function.

There are two models by which the host recognizes the graft: direct and indirect presentation (14). Direct recognition occurs when the host CD4 T cells respond to intact donor MHC class II on donor antigen presenting cells (APC). Possible APC include resident dendritic cells like Langerhan cells, Kupffer cells in the liver, or endothelial cells. Direct recognition may occur either in the draining lymph nodes of the host (donor APC migrate to the lymph node) or in the graft. Chemokines and the expression of adhesion proteins and

foreign MHC antigens by the endothelium of the graft mediate homing of T cells to the graft. Acute injury of the graft during transplantation may also increase tissue immunogenicity by increasing the expression of various molecules that are important in the immune response (15-20). The term indirect pathway refers to the ability of host CD4 T cells to recognize donor MHC as peptides in the MHC groove of host APC. Indirect recognition likely occurs in the host lymph nodes.

Whether cell-mediated or antibody mediated, the immune response to an allograft is T-cell dependent. Mice that are deficient in T cells do not reject grafts. Much of transplant immunosuppression has thus focussed on the T cell. Three signals are required for the full activation of a T cell (21). Signal 1 is provided by the engagement of the T cell receptor with MHC-peptide complexes. Costimulatory signals from the APC engaging receptors on the T cells provide signal 2. The major costimulatory molecules on the APC are B7-1 and B7-2, which bind to CD28. Activation of the T cell through signals 1 and 2 results in the production of cytokines such as interleukin (IL)-2. The engagement of IL-2 and other growth-promoting cytokines such as IL-15 with their receptors on the T cell provides signal 3 for cell division and clonal expansion. Activated T cells express CD40 ligand (CD40L) which can activate the APC by binding CD40 on the APC (22). The CD40:CD40L interaction is essential for immunoglobulin class

switching in the antibody response, as illustrated by the X-linked hyper-IgM immunodeficiency syndrome in humans and CD40 or CD40L-gene disrupted mice (23-25). CD40L is limiting for humoral responses to T cell-dependent antigens (26) but also has other roles in immune regulation, including T cell priming, differentiation, and effector functions (27). The requirement for CD40:CD40L interaction differs between CD8 and CD4 T cells and depends on the type of antigen presenting cell (APC) (28-30).

III. Cyclosporine and Tacrolimus

The goal of immunosuppressive therapies is to prevent rejection of the graft while minimizing the risks of oversuppression. Steroids were the first drugs to be used for immunosuppression. In 1951, Billingham *et al* demonstrated that the administration of cortisone could prolong skin graft survival in a rabbit model (31). The 6-mercaptopurine family of antimetabolites was discovered and developed by Elion and Hitchings (32;33). Schwartz and Dameshek discovered the ability of 6-mercaptopurine to repress graft rejection in 1959 and also experimented with its derivative azathioprine (34). In 1964, the combination of steroids and azathioprine became the first immunosuppressive regimen (35). Cyclosporine (CsA) and tacrolimus

(FK506 or FK) were the next major discoveries that revolutionized transplantation.

Cyclosporin A and Tacrolimus: Drug Discovery

In the early 1970s CsA, a hydrophobic and cyclic peptide, was isolated from culture broths of fungi *Tolypocladium inflatum* and *Cylindrocarpon lucidum* (Figure 1). The antilymphocytic properties of this 11 amino acid 1202.6 Da fungal metabolite were described by Borel *et al* working in Basel, Switzerland (36). CsA, first used clinically by Calne in 1978 (37), improved patient and graft survival by lowering the incidence of acute rejection in kidney, liver, and heart transplantation (38-42). The advantages of CsA over previous therapies included improved efficacy, lack of myelotoxicity, and reduced steroid requirements. In 1987 tacrolimus (FK506 or FK), an 822.05 Da immunosuppressive macrolide, was discovered in Tsukuba, Japan by Kino *et al* in the fermentation broth of *Streptomyces tsukubaensis*, and was introduced into clinics in 1989 (Figure 2) (43). Although they are structurally dissimilar, FK and CsA have similar immunosuppressive effects.

Inhibition of the immune response

The *in vitro* and *in vivo* immunosuppressive effects of CsA were first reported by Borel *et al* (36). CsA suppressed both humoral and cellular immunity in animals. CsA administered to mice inhibited direct and indirect hemolytic plaque-forming cells and hemagglutinin formation in a dose-dependent manner, prolonged survival of skin allografts, and decreased the incidence and symptoms of experimental allergic encephalomyelitis and Freund's adjuvant arthritis. CsA and FK were shown to suppress mixed lymphocyte reactions, antibody production, delayed-type hypersensitivity response, IL-2 secretion (43;44). Inhibition of IL-3 and 4, interferon (IFN)-γ, granulocyte-monocyte colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF)-α production have also been demonstrated (45). CsA and FK block progression from the G_0 resting phase to the G_1 activation phase in T cells, acting early after T cell stimulation. Treatment following expression of these early cytokine mRNAs fails to inhibit cytokine production.

Both drugs also block degranulation by mast cells, neutrophils, basophils, and cytotoxic T-lymphocytes (CTL) (46). Furthermore, CsA and FK block B cell division in the late activation phase (G1) of the cell cycle and treatment may occur as late as 24 hours after stimulation (47). The principal difference between CsA and FK is the

concentration or dose that is required for suppression of immune function. The immunosuppressive effect of FK is achieved at lower concentrations than CsA *in vitro*, *in vivo*, and clinically. For example, FK inhibited IL-2 production and mixed lymphocyte reactions at 20- to 50-fold lower concentrations and prolonged the survival of primary and secondary skin allografts at ~35-fold lower doses (48;49).

Pharmacokinetics

CsA and FK bind erythrocytes and plasma proteins, namely lipoproteins (CsA) and albumin (FK). Whereas the absorption of FK and the microemulsion formulation of CsA are bile-independent, some CsA formulations are bile-dependent. Both CsA and FK are extensively metabolized in the liver and gut by the cytochrome P450 3A4 (CYP3A4) system. Therefore, hepatic dysfunction alters drug clearance and half-life, increasing plasma drug concentrations and toxicity. CsA is excreted mainly by the biliary system. The half-lives for CsA and FK are 18 and 12 hours, respectively. Most metabolites of CsA and FK have very little, if any, immunosuppressive activity.

Drug monitoring is important for both CsA and FK because intra- and inter-subject variations make predictions of drug concentration on the basis of a given dose difficult. High performance liquid chromatography, monoclonal radioimmunoassay (RIA), and

monoclonal fluorescence polarization immunoassay (TDX) can be used to measure levels of CsA (50;51). Monoclonal RIA and TDX however give higher readings because of cross-reaction with CsA metabolites. The concentration of CsA in whole blood is approximately two-fold that in plasma. For detection of FK, various methods include enzyme-linked immunoabsorbent assay for plasma or whole blood and microparticulate enzyme immunoassay for whole blood can be used (52-56). Concentrations of FK in whole blood are approximately 10 to 20 times higher than in plasma.

Molecular Mechanism of Action

An important advancement in understanding the mechanism of action was the recognition that CsA blocked the induction of IL-2 mRNA and inhibited the activation of transcription of many cytokines in T cells (57;58). CsA was subsequently shown to bind to a ubiquitous and abundant set of proteins called cyclophilins (CyP) (59) while FK was shown to bind to an unrelated set of ubiquitous proteins called FK506 binding proteins (FKBPs) (60). Both CyPs and FKBPs were found to have rotamase or proline isomerase activity (60;61). Binding of CsA and FK inhibited the peptidyl-prolyl cis-trans isomerase or rotamase activity of immunophilins and it was initially believed that this inhibition was the basis for the immunosuppressive properties of these

two drugs (59;60). However, subsequent experiments showed that complete inhibition of isomerase activity was not necessary for complete inhibition of the immune response and that some cyclosporine analogs could bind and inhibit isomerase activity without inhibiting the immune response (62;63). These studies showed that the inhibition of the romatase activity was not related to the drugs' immunosuppressive mechanism.

The next major advancement came when both CsA:CyP and FK:FKBP complexes were found to bind to and inhibit the protein phosphatase 2B calcineurin (CN). Furthermore, the immunosuppressive activity of CsA and FK correlated with the inhibition of the enzymatic activity of CN by the drug: immunophilin complex (64-66). Crystallization of the FK:FKBP:CN ternary complex revealed that as the drug:immunophilin complex binds more than 10 Å away from the active site of CN (67;68). Thus FK:FKBP non-competitively inhibits CN phosphatase activity by sterically hindering the dephosphorylation of substrate. Although mutational studies have shown distinct binding interactions for CsA:CyP and FK:FKBP, the binding regions overlap and CsA is thus assumed to have a similar mechanism of action (Figure 3) (65;69). Based on the FK:FKBP:CN structure, a molecular model for the interaction of CN with CsA:CyP

has recently been proposed (70). As discussed in a later section, CsA and FK may also block the binding or docking of substrate to CN.

A rate-limiting enzyme in the calcium pathway, CN controls the phosphorylation of transcription factors important for the expression of immune-response genes, such as the nuclear factor of activated T cells (NFATC) (Figure 4)(71;72). However, it is not clear that the immunosuppressive actions of CsA and FK are explained entirely by the role of CN in NFATC regulation. As will be discussed in a latter section, CN regulates other molecules potentially relevant to T cell activation, such as elk-1, nitric oxide synthase (NOS), nuclear factor κ B (NFκB), and jun N-terminal kinase (JNK) (73-76).

P-glycoprotein

P-glycoprotein (PGP) is a protein encoded by the multidrug resistance (MDR) gene and functions as an adenosine triphosphate (ATP)-dependent pump capable of transporting different hydrophobic, cationic, or amphoteric substrates. PGP is vital in maintaining the integrity of the blood-brain, blood-testes, and blood-placenta barrier (77;78). PGP reduces the accumulation of drugs within cells and its over-expression is associated with multi-drug resistance (79;80). FK and CsA are both substrates for PGP and can reverse multi-drug

resistance. This activity is not dependent on its ability to induce immunosuppression (81;82).

Inhibition of CN activity rises and falls with CsA blood levels (83-85). The inhibition of CN activity of transplant patients on CsA therapy is rapidly reversible (86;87). There are at least two mechanisms by which CsA leaves the cell. One mechanism involves a rapid, PGP-dependent efflux that is only observed in cell lines expressing high levels of PGP and that can be blocked by competitive PGP substrates. There is also a slower, PGP-independent transport mechanism that requires the addition of extracellular CsA binding sites, such as erythrocytes. CsA may leave the cells by simple diffusion, not requiring active transport. Peripheral blood leukocytes (PBL) express relatively low levels of PGP. Thus the PGP-independent mechanism likely predominates. *In vitro* the recovery of CN activity is slow, likely because of the aqueous environment and the lack of extracellular binding sites (87). The recovery of CN activity *in vitro* does not require protein synthesis, is temperature-dependent, and is correlated with the efflux of CsA. Finally, although initially suggested to play an important role in CsA resistance, PGP expression in peripheral blood leukocytes is not a useful predictor of acute or chronic kidney graft rejection (88).

Toxicity

The toxicity of an immunosuppressive drug includes the general effects of interfering with the immune system (selected infections and malignancies) as well as the non-immune toxicities of the specific agent or class of agent. CsA and FK probably induce similar states of immunodeficiency and they share numerous nonimmune adverse effects (e.g. nephrotoxicity, uric acid increase). There are also immunodeficiency manifestations such as increased incidence of infections and malignant lymphomas. However, while neurotoxicity, diabetes, and alopecia are more prevalent during FK therapy, hypertension, gum hyperplasia, lipid abnormalities, hirsutism, and skin changes are more prevalent with CsA therapy (89). It has thus far not been possible to separate the therapeutic and nephrotoxic effects, suggesting that they are mechanistically similar (90). However, it is not clear whether all effects are CN-dependent. Both CN and immunophilins are ubiquitous and both drugs enter most cells and tissues. Two important questions remaining to be answered are what limits the toxicity of CsA and FK and what is the basis for their distinct side-effects.

IV. Calcineurin

Expression, Structure, and Regulation

First detected in skeletal muscle (91) and brain (92), CN is a calcium (Ca^{++})- and calmodulin (CaM)- dependent serine/threonine protein phosphatase ubiquitously expressed in mammalian tissues and is conserved from yeast to humans (93;94). Although characterized as a serine/threonine phosphatase, CN has also been shown to be capable of tyrosine dephosphorylation (95). CN is a heterodimer of two subunits: calcineurin A (CNA) and calcineurin B (CNB). Found tightly bound together, CNA and CNB can only be dissociated under denaturing conditions (96). While CNA is the catalytic and calmodulin-binding subunit, CNB is the Ca^{++}-binding regulatory subunit (93).

Human, rodent, Drosophila, and yeast sequences for CNA have been reported. Three separate genes encode CNA (58-64 kD): Aα, Aβ, and Aγ. Whereas CNAα and CNAβ are widely distributed, CNAγ is testis-specific (97). Functional differences between these isoforms are not well-characterized. With the exception of the N- and C-terminal tails, the three isoforms exhibit 83-89% identity over 90% of their sequence (98). As shown in Figure 5, CNA has four domains, one catalytic and three regulatory. The catalytic domain of CNA shows 40-50% similarity to protein phosphatases 1 and 2A. The regulatory domains of CNA consist of a CaM-binding domain (99), a CNB-binding

domain (100), and an autoinhibitory domain (AID) (101). The AID is near the C-terminus and was identified by limited proteolysis (102). Acting like a pseudosubstrate, this inhibitory domain folds over and covers up the catalytic site of CNA (101). CaM and CNB control the Ca^{++}-dependence of CN phosphatase activity. Although structurally similar, CaM and CNB are functionally different Ca^{++}-binding proteins. The binding of CaM is thought to displace the AID while the binding of Ca^{++} to CNB is believed to induce an allosteric change that alters the active site and augments its catalytic activity (103).

CNB has high affinity for Ca^{++}, but like other Ca^{++}-regulated proteins, is also regulated by Mg^{++} (93). Two isoforms of this 19 kD protein have been demonstrated. CNB1 associates with CNAα/CNAβ and CNB2 associates with CNAγ in the testes (104;105). CNB is most homologous with CaM in the four Ca^{++}-binding domains, which are often referred to as the "EF-hand" structure and which are found in most Ca^{++}-binding proteins (100). In the absence of CaM, Ca^{++} stimulates CN activity only at a low level. Addition of CaM with Ca^{++} produces a large increase in activity. The binding of Ca^{++} to CNB modulates the interaction with and activation of CN by CaM. The V_{max} is increased by Ca^{++} and CaM without any effect on the K_m for the substrate (103).

Sustained and elevated levels of intracellular Ca^{++} are required to activate CN. The mechanism by which this is achieved during T cell activation is intricate. Activation of PLC-γ1 results in the formation of two important second messenger molecules, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses through the cytosol and activates its receptor on the membrane of the endoplasmic reticulum (ER). Binding opens the Ca^{++} channels and releases a small burst of Ca^{++} from ER storage sites. This initial rise in intracellular Ca^{++} is insufficient to induce gene expression, cell proliferation or differentiation and is limited by inactivation of IP_3 by dephosphorylation and cytosolic extraction of Ca^{++} by the membrane Ca^{++} ATPase pump. The transient burst in intracellular Ca^{++} activates the inwardly rectifying intracellular Ca^{++} release activated Ca^{++} (CRAC) channel (106). The increased entry of Ca^{++} must be balanced by the increased outflux of K^+ through type *n* voltage-gated K^+ channels (107;108). This large and sustained increase in intracellular Ca^{++} is required to activate CN. Studies by Berridge *et al* suggest that this prolonged Ca^{++} stimulus is actually a sustained series of Ca++ spikes rather than a constantly high level of Ca^{++} (109).

In addition to Ca^{++}, various other metal ions also directly regulate CN phosphatase activity (e.g. Mg^{++} and Ni^{++}). The ability of these other metal ions to stimulate CN activity depends on the

concentration, pH, and substrates used (93;110;111). Zn^{++} and $Fe^{++/3+}$ in the active site are thought to serve for the catalytic reaction (112). Recent studies suggest that CN may be modulated by the intracellular redox potential (98;113;114). While oxidants such as hydrogen peroxide inhibit the phosphatase activity of CN, antioxidants such as ascorbate increase phosphatase activity. CN is sensitive to oxidative inactivation through its Fe-Zn active centre and this can be prevented by the enzyme superoxide dismutase (113). These may also be mechanisms by which CN is regulated *in vivo* and are important to consider when assaying enzyme activity *in vitro*.

Endogenous protein regulators

A number of endogenous cellular protein regulators of CN have been discovered. These proteins interact with CN and regulate its subcellular targeting and/or phosphatase activity. One of the first to be identified was a 79-kDa protein kinase A anchoring protein (AKAP79). AKAP79 is an anchoring protein that is enriched in neurons and present in T cells. Able to bind the membrane via its amino terminal basic region, AKAP79 is believed to anchor PKA, CN, and PKC to specific microenvironments (115-118). CN binds AKAP79 at a site distinct from that for protein kinase C (PKC) or protein kinase A (PKA). AKAP79 binds CN at a site distinct from that for the drug:immunophilin

complex (118;119). Binding of AKAP79 noncompetitively and specifically inhibits the phosphatase activity of CN. AKAP79 thus localizes CN in an inactive state and over-expression inhibits NFATC2 dephosphorylation and PMA/ionomycin-induced NFAT activity (119).

Bcl-2, a cytoplasmic protein found on the membranes of the mitochondria and endoplasmic reticulum, has also been shown to regulate CN. Bcl-2 inhibits cell death induced by Ca^{++} signaling and growth factor withdrawal; apoptosis induced by expression of a constitutively active form of CN can be abrogated by bcl-2 expression (120-122). In BHK cells co-transfected with bcl-2 and in T- and B-cell lines expressing high levels of bcl-2, a physical interaction between bcl-2 and CN can be detected (123). CN interacts with the BH4 domain, the α-helical domain present in the anti-apoptotic members and absent in the pro-apoptotic members of the bcl-2 family. Unlike AKAP79, bcl-2 does not inhibit CN phosphatase activity. It anchors CN to cytoplasmic membranes and prevents CN from escorting NFATC into the nucleus, where it is required to protect NFATC from rephosphorylation (123;124). The site of bcl-2 interaction on CN is distinct from that for the drug:immunophilin complex; CsA is still able to bind and inhibit the phosphatase activity of CN bound to bcl-2. The pro-apoptotic members of the bcl-2 family can also regulate the interaction between bcl-2 and CN (123). Notably, an interaction

between bcl-2 and CN was not detected in the cell line expressing low levels of bcl-2. Thus it is not known whether this interaction occurs in normal cells.

Cain/cabin 1 was isolated from screens of rat hippocampal or mouse T-cell cDNA libraries (125;126). This 240 kD phosphoprotein binds and noncompetitively inhibits CN phosphatase activity. The interaction between cain/cabin 1 and CN is PKC dependent. Overexpression inhibits the transcriptional activation of CN-responsive elements in the IL-2 promoter and blocks the dephosphorylation of NFATC upon T-cell activation. Cain/cabin 1 has also recently been shown to bind and sequester in an inactive state the transcription factor myocyte enhancer factor (MEF) 2, which itself is also regulated by CN-dependent mechanisms (127).

Calcineurin B homologous protein (CHP) is another potential regulator of CN *in vivo*. Specifically bound to the Na^+-H^+ exchanger NHE1, CHP is important in the stimulation of exchange activity by growth factors (128). Overexpression in Jurkat and HeLa cells resulted in inhibition of the nuclear translocation and transcriptional activity of NFATC. CN phosphatase activity inhibited 50% in these cells and CHP also inhibited purified CN *in vitro* in a dose-dependent manner (129).

More recently, myocyte-enriched calcineurin interacting proteins (MCIPs), the products of DSCR1 (Down syndrome critical region) and ZAKI-4, have been postulated to modulate CN signaling in humans. Overexpression inhibits CN activity in yeast and mammals; MCIPs also bind CN *in vitro* and inhibit its phosphatase activity. These proteins are thought to be negative feedback regulators of CN and their expression is up-regulated during muscle differentiation (130;131).

CN outside the immune system

The importance of CN in the immune system is best illustrated by the immunosuppressive effects of CsA and FK (as discussed in a previous section). Considering its ubiquitous expression, the elucidation of roles for CN outside of the immune system is not surprising. A better understanding of CN in these other systems will help to better understand the actions of CN inhibitors as well as help to develop more specific and less toxic immunosuppressive agents.

Recent studies have implicated a role for CN in cardiac hypertrophy. Cardiac hypertrophy is a compensatory mechanism that maintains cardiac output in response to conditions such as hypertension and myocardial infarction. Sustained, it can lead to heart failure and death. Transgenic mice expressing active forms of CN develop cardiac hypertrophy and heart failure. Mice transgenic for an

active form of NFATC4 also develop cardiac hypertrophy, suggesting that CN acts through activation of NFATC4. Activated NFATC4 interacts with GATA4 to induce genes leading to the hypertrophic response. CN may also act by activating MEF-2, a myogenic transcription factor which up-regulates many of the genes during hypertrophy (132;133). Furthermore, CN has been shown to play a role in cardiomyopathy resulting from defects in contractility caused by sarcomeric dysfunction (134). The hypertrophic response induced by angiotensin II and phenylephrine *in vitro* through activation of NFAT can be inhibited by CsA and FK (135). However, neither CsA nor FK could prevent left ventricular hypertrophy induced by aortic banding, suggesting that CN is not the sole pathway for the hypertrophic response (136;137).

In the nervous system, CN is necessary for normal neural functions and may also function in pathological states such as ischemic or traumatic neuronal injury. CN regulates Ca^{++} flux (via IP_3-R), decreases glutamate release (via synapsin, dynamin), increases stability of microtubules (via tau, MAP-2, tubulin) and increases NO (via NOS, neurogranin, neuromodulin) (74;138-140). Both ischemia and trauma result in glutamate release and increases in intracellular Ca^{++}. Inhibition of NO production through inhibition of CN has been suggested to underlie the neuroprotective effects of CsA and FK in

ischemic injury. Long-term depression (LTD) is an activity-dependent decrease in synaptic efficacy that permits neural networks to store information more effectively. CN is thought to potentiate LTD by dephosphorylating and inactivating inhibitor-1, an inhibitor of protein phosphatase 1 (PP1) (141). PP1 is then activated and dephosphorylates target proteins such a cAMP-responsive element binding protein (CREB) (142). Phosphorylated CREB promotes the activation of genes for long-term memory. CREB may also be a direct substrate of CN (143). The basis for the neurotoxic effects of the CN inhibitors is not understood. Some possibilities will be discussed in chapter 6.

Recent studies have also implicated a role for CN in the skeletal system. Muscle growth and regeneration are important adaptive responses in health and disease states. There are three major types of muscle fibers: slow-oxidative (type 1), fast oxidative (type IIa), and fast-glycolytic (type IIb). The two main differences between them are their speed of contraction (slow/fast) and the enzymatic machinery used for ATP formation (oxidative/glycolytic). Size, strength, and speed are determined mainly by the amount and type of contractile protein incorporated (144). Adaptation occurs over time in response to workload and pathological stimuli. Increased motor nerve activity from electrical pacing or exercise training induce fast-to-slow fiber

transformation while decreased motor nerve activity from certain disease states, hypogravity, or physical inactivity induces slow-to-fast fiber transformation. CN and NFATC are abundant in skeletal muscle and have been implicated in myofiber specialization (145). CN was found to transcriptionally activate promoters for myoglobin (Mb) and troponin I slow (TnIs), which are expressed selectively in slow oxidative skeletal muscle fibers, but not muscle creatine kinase, which is expressed mainly in fast, glycolytic myofibers. Transactivation of these slow-fiber-specific promoters requires collaboration among multiple transcription factors, including NFATC and MEF2 (146). Recently, CN has also been implicated in skeletal muscle growth (147). Activation of GATA-2 and NFATC1 is thought to underlie the CN-mediated hypertrophy induced by insulin-like growth factor-1 (IGF-1)(147). CsA and FK treatment has been shown to prevent muscle hypertrophy and fast-to-slow fiber transformation during functional overload, and may underlie muscle weakness in post-transplant patients (148).

CNAα-deficiency

The immune systems of mice lacking CNAα were characterized by the Seidman group in early 1996 (149). The composition and distribution of T and B cells were normal in thymus, spleen, lymph nodes, and bone marrow, suggesting that CNAα is not required for

normal development of T and B cells. CNAα-/- mice responded normally to challenge with TNP-OVA; however, when re-stimulated with OVA *in vitro*, T cells harvested from lymph nodes of CNAα-/- mice proliferated less and secreted less IL-2, IL-4, and IFN-γ than T cells from wild-type mice. This defect could not be corrected by addition of normal APCs or IL-2 to the *in vitro* culture. However, stimulation with polyclonal mitogens, such as PMA plus ionomycin, ConA, or anti-CD3ε antibody showed no functional differences between CNAα-/- and wild-type T cells *in vitro*. T cells from CNAα-/- mice were also more sensitive to inhibition by CN inhibitors CsA and FK. More studies of the CNAα-deficient mouse are necessary to understand the discrepancy between the *in vitro* and *in vivo* findings.

As assessed by the CN phosphatase assay, there was >65% reduction of CN activity in T cells from CNAα-/- mice (149). Similarly, this laboratory found an 82-88% reduction of CN activity in the heart, brain, kidney, and spleen of CNAα-/- mice (Kung *et al* submitted). These observations however differed from other studies that suggested that the predominant isoform in T- and B-lymphocytes was CNAβ (150;151). It may be that CNAα is the predominant activity detected in the CN assay. However, this laboratory has assessed CN activity in whole cell lysates (supernatant plus pellet) and compared it to supernatants and found no increase in activity. Although functional

loss cannot be ruled out, physical loss of CNAβ during extraction is unlikely to explain the discrepancy.

The neurological characterization of the CNAα-/- mouse is consistent with the hypothesized role of CN in the brain. These mice were observed to have deficits in learning and memory (152). Hyperphosphorylated tau (τ) was found to accumulate in the brains of CNAα-deficient mice. τ is a protein associated with the microtuble and functions to stabilize microtubules and promoting their assembly by forming cross-bridges between microtubules and between microtubules and neurofilaments. Hyperphosphorylation of τ changes its physical and functional properties, decreasing its activity. Synaptic depotentiation was also shown to be completely abolished (153).

This laboratory has examined the basic morphology of brain, thymus, liver, heart, kidney, and spleen of 4-5 month-old CNAα-deficient mice by hematoxylin and eosin (H & E) staining and Periodic Acid-Schiff (PAS) staining. No abnormalities were observed in any of the organs except for the liver and spleen. Slightly more mitotic bodies in the liver and more prominent germinal centers in the spleen were noted however.

Mice with catalytically inactive CNAα (dominant negative CN or dnCN) were generated last year (154). CN activity in these mice was reduced by approximately 50% although the amount of CNAα protein T

cells was two-fold that of normal littermates. No impairment in T cell development was observed but there was a slight decrease in TCR-induced proliferation and IL-2 production. Th2 cell development was also shown to be impaired while Th1 was unaffected. This study suggested that CN controlled Th2 cell development in part by affecting the IL-4R signaling complex; however, this hypothesis was based on data other than the dnCN mice. That is, the authors did not test the hypothesis in dnCN T cells.

V. NFATC Family of Transcription Factors

CN directly and indirectly modulates the activity of a number of intracellular proteins, including NFATC, elk-1, IP_3R, NOS, NFκB, JNK, and a number of neuroproteins (73-76;138-140;155;156). The members of the NFATC family of transcription factors are the most well characterized substrates of CN.

Expression and Structure

Rao *et* al have recently reviewed the first four members of the NFATC family identified (157). With more than one name for each member, the nomenclature for this area is somewhat confusing (Table 1). Whereas NFATC1 and NFATC3 are tightly restricted to the lymphoid system in the adult system, the expression of NFATC2 and

NFATC4 is fairly ubiquitous. There are two major regions of sequence homology in all NFATC proteins (Figure 6): the DNA-binding domain (DBD) and the NFAT homology region (NHR) (158;159). Located between amino acid residues ~400 and ~700, the DBD is the region of highest homology and shows similarity to the DNA binding domains of the Rel-family of transcription factors (158;160). The NHR, ~300 amino acids and just N-terminal to the DBD, is found only in NFATC family proteins and is the region where CN binds (161). There are at least two distinct calcineurin binding sites on the NFAT regulatory domain (162;163). Also within the NHR are a serine-rich region and a SP repeat. Dephosphorylation of these serines by CN results in the exposure or activation of nuclear localization sequences (NLS) that direct the nuclear import of NFATC (124). Export of NFATC from the nucleus is thought to depend on a nuclear export sequence (NES) (164).

A recently cloned NFAT-related protein, NFAT5, differs from NFATC1-4 in its structure, DNA binding, and regulation. NFAT5 is similar in its Rel-like DNA-binding domain. However, it differs in that it does not cooperate with Fos/Jun at NFAT:AP-1 composite sites and is constitutively nuclear regardless of CN activation (165).

NFATC deficiency and NFATC function

NFATC knockouts have provided valuable information about the role of the various NFATC members in the immune response. The NFATC1 knockout showed hypoproliferation of peripheral T- and B-cells in response to mitogens, impaired T-cell development, and a selective decrease in IL-4 (166). In contrast, the NFATC2 knockout showed modest splenomegaly, T- and B-cell hyperproliferation, impaired induction of FasL, and a moderate increase in Th2-type cytokines (IL-4, 5,13) (167). Mice deficient in NFATC3 have a defect in T cell development, increased apoptosis of double-positive thymocytes, increased activation of peripheral T cells, and impaired expression of bcl-2 (168). The double knockout of NFATC3 and NFATC2 showed massive splenomegaly and lymphadenopathy, a marked increase in mast cells and eosinophils, hyperactivated T- and B-cells, impaired expression of FasL, and a dramatic increase in Th2-type cytokines and IgG1 and IgE (169).

The phenotypes of the NFATC deficient mice are consistent with *in vitro* studies demonstrating the involvement of CN and NFATC in the expression of cytokines and other immunologically relevant genes (157;170;171). There is redundant but selective utilization of NFATC proteins (172;173). Interestingly, two human male siblings with severe combined immunodeficiency were determined to have defective NFATC DNA-binding activity in their T cells (174). It was subsequently

shown that the defect was the result of an impairment in the dephosphorylation and nuclear translocation of NFATC (175). Evidence from this study also suggested that the duration of NFATC nuclear localization could determine the pattern of cytokine expression. In addition to their roles in the immune system, NFATC family members have also been implicated in many other processes, such as Gq-protein-coupled receptor signaling, adipogenesis, neuropoiesis, slow-to-fast skeletal fiber transformation and cardiac morphogenesis (146;176-180).

Regulation

NFATC is found in its phosphorylated state in the cytosol in resting cells. Upon activation by an increase in intracellular Ca^{++}, CN binds to and dephosphorylates NFATC (181). JNK can inhibit the targeting of CN to NFATC1 by phosphorylating and inactivating the CN targeting domain of NFATC1 (182). Dephosphorylation of NFATC by CN is thought to unmask a nuclear localization sequence (NLS), resulting in the nuclear translocation of the NFATC-CN complex (164).. CN is required to escort and to protect NFATC from constitutively active kinases such as glycogen synthase kinase-3, casein kinase 1, JNK, and MEKK1 (124;183;184). These kinases bind and

phosphorylate functionally important residues that regulate the subcellular localization of NFATC (185).

Once in the nucleus, NFATC forms cooperative complexes with Fos and Jun on DNA to induce the transcription of such genes such as IL-2 and IFN-γ. The crystal structure of the DNA-binding domains of NFATC, Fos, and Jun showed a tight association of the three transcription factors resulting in cooperative binding to DNA (186). When Ca^{++} signaling ends, CN dissociates from NFATC and NFATC kinases rephosphorylate the NLS sequence (124;183;184). The nuclear export of NFATC is mediated by nucleocytoplasmic shuttling factors such as Ran and crm1 (187). Crm1 is unable to bind until CN dissociates from NFATC.

Selective inhibition of NFATC

The activation of NFATC by CN can be specifically inhibited by peptides that interfere with the targeting of CN to NFATC (72). SPRIEIT peptides spanned the CN targeting site of NFAT and selectively interfered with the physical interaction between CN and NFATC. They inhibited the ability of CN to bind to the NFATC without affecting CN phosphatase activity towards other substrates (188). More recently, VIVIT peptides were shown to be 25 times more effective than SPRIEIT peptides at inhibiting the binding of activated

CN to NFATC. VIVIT peptides are high affinity CN docking sites that were selected from combinatorial peptide libraries (189). The selective inhibition of NFATC activation is postulated to be less toxic than the inhibition of general CN phosphatase activity (72). However, the possibility that the toxicities of CsA and FK are due to inhibition of NFATC remains. As roles for NFATC family members outside the immune system are elucidated, the potential toxicities of selective NFATC inhibition might be predicted.

VI. Immunophilins

Immunophilins are a family of proteins originally identified by their ability to bind CsA or FK. While CyPs bind CsA, FKBPs bind FK and rapamycin. Although structurally dissimilar, both CyPs and FKBPs have peptidy-prolyl *cis/trans*-isomerase activity. Immunophilins may be involved in protein trafficking or refolding. Although unrelated to their immunosuppressive activities, all three drugs inhibit the romatase activity of their respective binding proteins.

Cyclophilin A (CyPA) was the first CyP to be identified (190); CyPB, CyPC, CyPD, CyP40, NK-TR, and ninA have since been identified. CyPs range in molecular mass (from 18 to 150 kD) as well as their affinity for CsA. Highly conserved throughout evolution, CyPs are expressed in most tissues. The first FKBP to be identified was

FKBP12 (60). Named for their molecular weights, FKBP12.6, FKBP13, FKBP25, FKBP33, FKBPr38, FKBP51, and FKBP52 range from 12 to 52 kD.

Although all immunophilins can bind their respective drug, not all drug:immunophilin complexes can inhibit CN. Rapamycin (sirolimus) and its analog SDZ RAD (everolimus), for example, bind FKBPs but the drug:immunophilin complex does not inhibit CN. CyPA, CyPB, FKBP12, FKBP12.6, and FKBP51 are the only ones that form active drug:immunophilin complexes which inhibit CN in the cell (191-193). Some immunophilins such as FKBP13 and FKBP25 form inactive drug:immunophilin complexes that are neither able to bind nor inhibit CN (194). Other immunophilins such as CyPC form active drug:immunophilin complexes but are inactive because they are sequestered away from CN by their subcellular localization (191). The significance of these inactive immunophilins in relation to the action of FK and CsA is not clear. They may reduce the amount of drug available for the active immunophilins or act as reservoirs.

VII. Transforming Growth Factor-β

Transforming growth factors (TGF) - β are multifunctional growth factors with roles in cell proliferation, lineage determination, cell motility, cell adhesion, and cell death. There are three mammalian

isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3. TGF-β isoforms are ubiquitously and differentially expressed in tissues. There is no phenotypic overlap of the isoform-specific knockouts, suggesting distinct roles for each isoform. Mice deficient in TGF-β1 have an excessive inflammatory response and massive leukocytic infiltration in several organs (e.g. heart, lung, liver). These mice die by the fourth week of severe wasting (195;196). TGF-β2-deficient mice have a wide range of developmental defects (e.g. heart, lung, eye) (197) and mice lacking TGF-β3 have cleft palate and postnatal defects in the conducting airways of the lung (198).

TGF-β's are secreted as small or large latent TGF-β complexes. Small latent TGF-β consists of the active TGF-β and its prodomain, the TGF-β latency-associated peptide (LAP) (199-201). Early in the secretory pathway, two chains of small latent TGF-β associate to form a disulfide-bonded dimer (201-203). TGF-β is cleaved from LAP by furin-like processing endoproteinase (204;205) but LAP remains associated with TGF-β by non-covalent interactions (201;206;207). TGF-β contains no carbohydrate but LAP is glycosylated at multiple asparagine residues. The small latent TGF-β complex is secreted very slowly and a significant portion is retained in the Golgi (208).

Large TGF-β complexes contain the small latent TGF-β and high-molecular-weight glycoproteins that associate with LAP (209-211). These glycoproteins are called latent TGF-β binding proteins (LTBP) and are disulfide linked to LAP (209). Although not required for latency, LTBPs are important in the processing, secretion, and targeting of TGF-β (203;212-217). Association of the small latent complex with LTBP results in rapid secretion of the large latent TGF-β complex (198;203;213). The majority of secreted LTBP however is not associated with small latent TGF-β (203;213;214;218) so LTBPs likely also serve as structural proteins of the extracellular matrix (213;217;219). LTBPs contain a core of 15-19 EGF-like repeats, three eight-cysteine motifs resembling fibrillin, and a hybrid domain. In other extracellular proteins, EGF-like repeats mediate protein-protein interactions (220;221).

Promoter region of TGF-β1 has been characterized. It has binding sites for AP-1 and Egr-1 transcription factors but does not contain TATA or CAAT boxes (222). Transformation with several oncogenes such as c-Ha-Ras, src, jun, fos, abl, and ras upregulate the expression of TGF-β1 (222-227). The AP-1 site mediates upregulation in response to TGF-β1 or phorbol ester (228-230) whereas Egr-1 site mediates induction by NGF (224). The promoter for TGF-β2 contains AP-1 and AP-2 sites, a cAMP regulatory element, and a TATAA

consensus start site (231). TGF-β2 can be induced by the retinoblastoma protein through transcription factor ATF-2 (232;233) and by retinoic acid (234). The TGF-β3 promoter region contains a TATA box, a cAMP response element, a 3XTCCC motif, and an AP-2 site. Forskolin induces TGF-β3 through the cAMP response element while the 3XTCCC motif is thought to regulate tissue-specific gene expression. The AP-2 site is not functional (235;236). TGF-β1 and TGF-β3 are also controlled translationally as the 5' untranslated regions suppress the translation of heterologous mRNAs (237;238). Latent TGF-β can be activated *in vitro* by extreme pH (239;240), proteases (241;242), cell co-culture (plasmin dependent) (243), thrombospondin (244), and glycosidases (245). Gamma radiation of mice has been demonstrated to activate latent TGF-β *in vivo* (246;247) and drugs such as retinoids (234), vitamin D (248) and glucocorticoids (249;250) can also induce activation in cell culture. Activation may involve (1) release of TGF-β1 from matrix, (2) activation of TGF-β1, (3) negative regulation by shedding of betaglycan from cell surface.

The TGF-β receptor (TβR) complexes are heterotetrameric, consisting of two type I (TβR-I) and two type II (TβR-II) receptors. Both TβR-1 and TβR-2 are N-glycosylated, have 10 or more cysteines, and have serine/threonine kinase domains (251-253). TβR-1 is required for

TGF-β signaling and determines signal specificity but cannot bind TGF-β independent of TβR-II. The assembly of the heteromeric complex is initiated by binding of the ligand dimer to two TβR-II's and is followed by the recruitment of two TβR-I's into the ligand-TβR-II complex. TβR-II then serine/threonine phosphorylates TβR-I at Ser165, resulting in its activation. Activated TβR-I then phosphorylates a receptor regulated SMAD (rSMAD, e.g. SMAD 2 or 3) in their MH2 domain. The phosphorylated rSMAD subsequently associates with a collaborating SMAD (co-SMAD, e.g. SMAD 4) and this SMAD complex then translocates to the nucleus where it mediates the transcriptional induction of the target gene (recently reviewed in (254)). There are also accessory (type III) receptors for TGFβ: betaglycan and endoglin. These receptors have no intrinsic signaling activity but are thought to regulate the access of the ligand to the signaling receptors (255;256).

TGF-β1 has recently been proposed to be a key mediator of the immunosuppressive and fibrogenic effects of CsA and FK. A multifunctional and ubiquitous growth factor, TGF-β1 is produced by many cell types including T cells. TGF-β1 inhibits T- and B cell proliferation, prevents cytotoxic T cell generation, inhibits Ig secretion, and is fibrogenic (257). TGF-β transgenic mice show evidence of nephrotoxicity, glomerulosclerosis, and fibrosis of kidney (258;259).

The phenotype of the transgenic mice is similar to the toxicities observed in transplant patients on CN-inhibitor therapy.

VIII. The Big Question: How do CsA and FK really work?

CsA and FK have been cornerstones for successful organ transplantation in the last two decades. The identification of CN as the direct target for both these drugs was a major breakthrough in understanding their mechanism of action. However, a number of questions remained and new questions were raised. Although qualitatively similar, CsA and FK are quantitatively distinct. Lower concentrations and doses of FK than CsA are needed *in vitro* and *in vivo*. Furthermore, these drugs have been shown to enter almost all cells and tissues. CN and the drug binding proteins are ubiquitously expressed. What determines the tissue-specific effects of CsA and FK? The objective of this thesis was to determine the molecular basis for the quantitative differences between CsA and FK and to better understand the mechanism and the tissue-specific effects of these drugs *in vivo*.

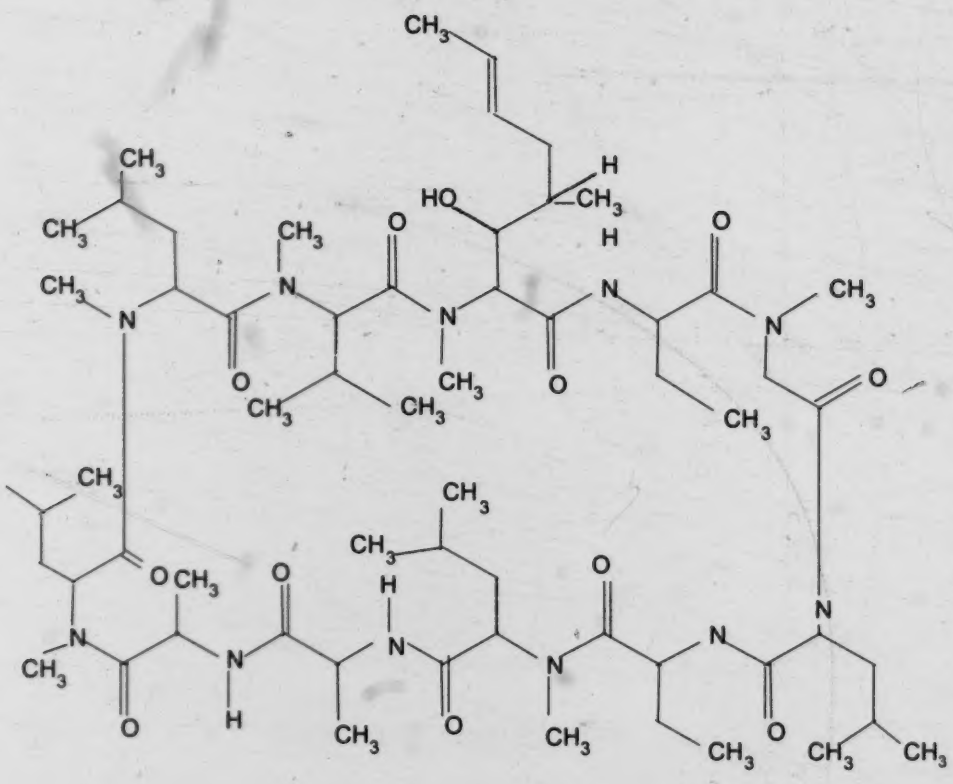


Figure 1. Structure of cyclosporine (CsA). Molecular weight is 1202.6 Da.

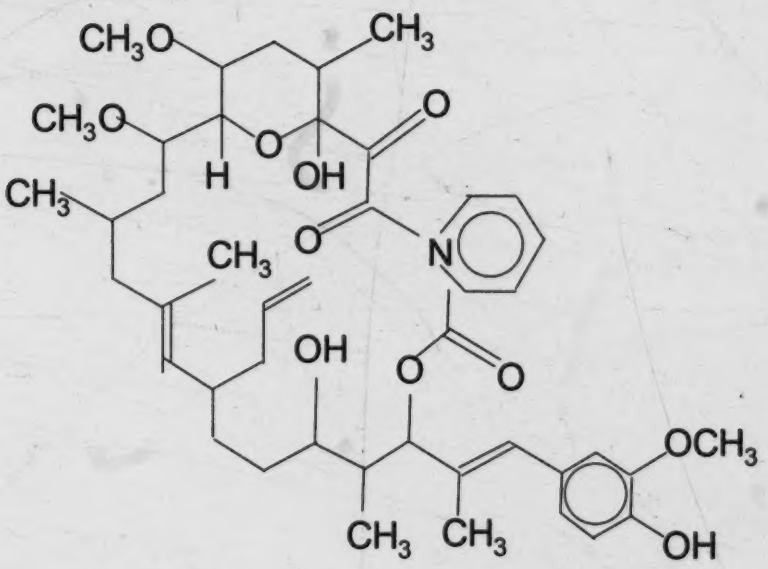


Figure 2. Structure of tacrolimus (FK506 or FK). Molecular weight is 822.05 Da.

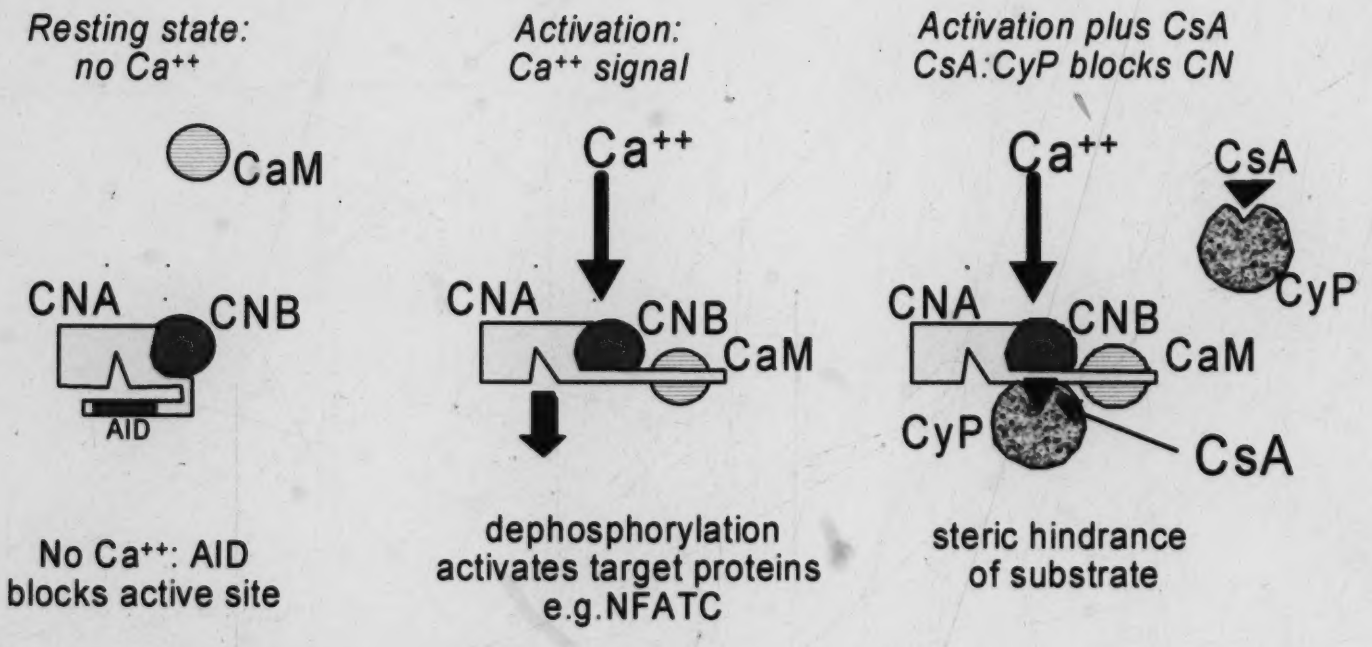


Figure 3. Mechanism of calcineurin (CN) inhibition by cyclosporine (CsA). In the resting state, there is low intracellular calcium and the autoinhibitory domain (AID) covers up the active site of CN. Following activation, there is an increase in intracellular calcium that results in the displacement of the AID and subsequent activation of CN. Treatment with CsA results in the formation of a drug:immunophilin complex (CsA:CyP) that binds to and noncompetitively inhibits CN phosphatase activity.

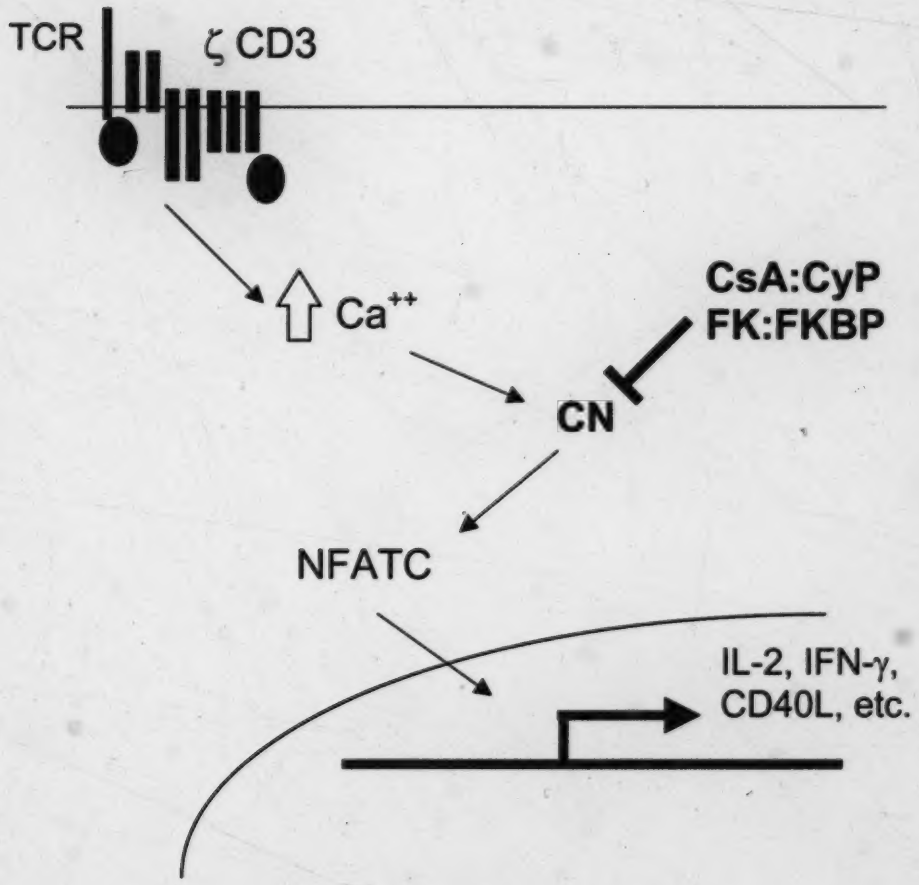


Figure 4. Inhibition T-cell activation by CsA and FK. A simplified diagram of the calcium pathway in T-cell activation. CsA and FK bind to their respective binding proteins (CyP and FKBP, respectively) and these drug:immunophilin complexes bind and inhibit CN, a rate-limiting enzyme in this pathway.

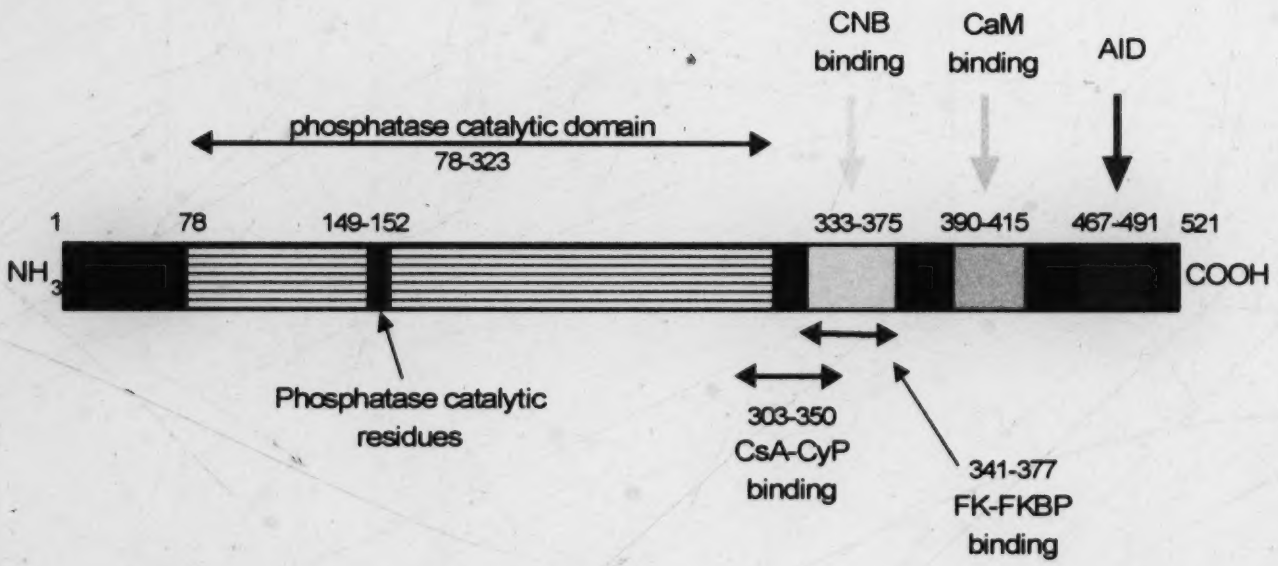


Figure 5. Structure of human CNAα. The catalytic domain, calmodulin (CaM) binding domain, calcineurin B (CNB) binding domain, and autoinhibitory domain (AID) are shown. The binding sites for the CsA:CyP and FK:FKBP complexes are also shown.

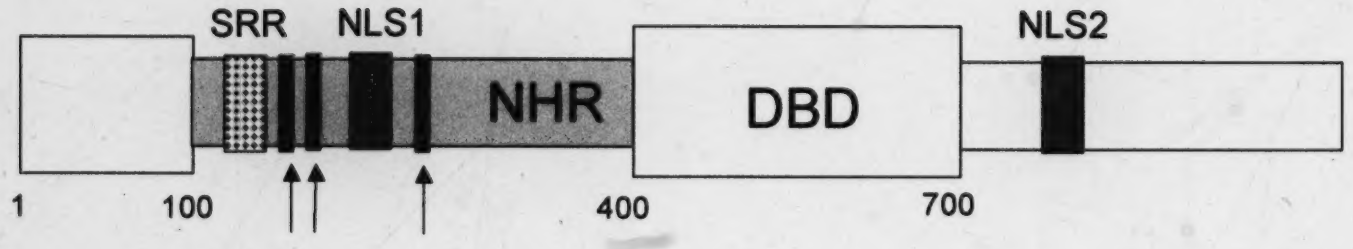


Figure 6. General map of the NFATC family. The two major regions of sequence homology are shown: NFAT homology region (NHR) and DNA binding domain (DBD). The first nuclear localization sequence (NLS1) is located in the NHR while the second (NLS2) is closer to the carboxy terminus. SRR indicates serine-rich regions (SRR). Arrows indicate SP repeats.

Table 1. NFATC Family: Nomenclature and Function

Agreed Upon	Also Known As	Immune Function
NFATC1	NFAT2, NFATc	Th2 activator
NFATC2	NFAT1, NFATp	Th2 repressor
NFATC3	NFAT4, NFATx	T cell generation and survival
NFATC4	NFAT3	none

Chapter Two

Materials and Methods

I. **Human samples.** All volunteers and patients described gave informed consent and the studies were approved by the Health Research Ethics Board (University of Alberta).

II. **Mice.** Female mice (BALB/c, A.Ly 6.2) aged 6-8 weeks were obtained from the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta, and were maintained in the HSLAS animal colony and kept on acidified water (2 mM HCl). All experiments were governed by approved animal care protocols.

III. **Cell suspensions.** For peripheral blood leukocytes (PBL), buffy coats (Canadian Blood Services, Edmonton, Canada) or whole blood were collected from healthy volunteers and PBL was isolated by standard Ficoll-Hypaque (Amersham Pharmacia Biotech, Baie d'Urfe, PQ) gradient centrifugation. For spleen cell suspensions (SCS), freshly harvested spleens from mice were forced through a metal sieve in PBS. Contaminating erythrocytes in PBL or SCS were lysed using hypotonic lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 300 μM EDTA) for about 5 minutes. Cells were then washed twice with PBS. Jurkat T cells, a human leukemic lymphoblast cell line, were obtained from the American Type Culture Collection (Rockville, MD).

IV. T cell enrichment. Human T cells were purified using commercial immunocolumns (Cellect, Biotex Laboratories, Edmonton, Canada) and were >95% pure. Enriched populations of mouse T cells were obtained by loading 50 million spleen cells onto a nylon wool column for 30 minutes at 37°C and eluting with about 10 mL of warm RPMI with 10% fetal bovine serum.

V. Culture conditions. For *in vitro* studies, cells were kept in a humidified chamber at 95% humidity, 37°C, and 5% CO_2. Cell culture medium was RPMI 1640 (GIBCO BRL, Gaithersburg, MD) with 10% pooled human serum (PHS; NABI, Miami FL) for human cells or 10% fetal bovine serum (FBS, GIBCO BRL, Gaithersburg, MD) for mouse cells. In TGF-β studies, x-vivo 15 serum free media (BioWhittaker, Walkersville, Maryland) or RPMI with 5% PHS plus 0.2 mM glutamine, 100 000 units/L penicillin G, and 100 mg/L streptomycin sulfate was used.

VI. *In vitro* stimulations. Cells in culture were stimulated with the calcium ionophore ionomycin (CalBiochem, San Diego, CA), phorbol myristate acetate (PMA, Sigma Chemical Co., Oakville, ON), or anti-CD3 (hamster). The duration of stimulation and concentrations were as indicated. Control groups were treated with the appropriate solvent.

VII. Cyclosporine (CsA) and Tacrolimus (FK).

a) *In vitro studies.* CsA (kindly provided by Novartis Canada) was initially dissolved in ethanol and Tween 80. The concentration of CsA was determined by HPLC and the stock solution was stored at 4°C. FK (kindly provided by Fujisawa Canada) was initially dissolved in ethanol. The concentration was determined by high performance liquid chromatography (HPLC) and the stock solution was stored at –70°C. Dilutions of CsA and FK were made in culture media or assay buffer.

b) *In vivo studies.* CsA (Neoral®, the oral formulation) was diluted in a commercial brand of olive oil and administered in 0.1 mL by oral gavage as indicated. FK (kindly provided by Fujisawa Canada) was initially dissolved in ethanol and the concentration determined by HPLC. This solution was then diluted in PEG400 (Sigma Chemical Co., Oakville, ON) and the desired dose was administered in 0.1 mL by oral gavage. Animals in control groups were given diluent alone.

c) *FK levels.* Levels for FK were determined by liquid chromatography/mass spectrometry (LC/MS, Isotechnika, Edmonton, Canada) for mouse tissues or by fluorescence polarization immunoassay (University Hospitals Laboratories, Edmonton, Canada) for patient samples.

VIII. Tissue extracts.

a) *CN activity.* Tissues were harvested, weighed, and homogenized with a polytron on ice in lysis buffer (50 mM Tris-HCl pH 7.5, 0.5 mM DTT, 50 μg/mL PMSF, 50 μg/mL soybean trypsin inhibitor, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM EDTA, and 0.1 mM EGTA). The volume (mL) of lysis buffer used for homogenization was tissue weight (g) X 8. Tissue homogenates were further diluted 1/4 (spleen, kidney, heart), 1/2 (liver), or 1/8 (brain) for the assay.

b) FK drug levels. Tissue were harvested, weighed, and pooled (n=4). They were then homogenized in PBS (volume (mL)=pooled tissue weight (g) X 3) and supernatants were stored at -70°C until analysis.

c) *Western analysis.* Tissues were harvested, weighed, and homogenized with a polytron on ice in RIPA lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 100 μg/mL PMSF, 30 μg/mL aprotinin, 1 mM sodium orthovanadate). Homogenates were then centrifuged twice at 13000 rpm for 10 min and the supernatant was frozen away at -20°C. For cell suspensions, whole cell extracts were prepared by resuspending cells in lysis buffer containing 5 mM Tris, pH 8, 25 mM NaCl, 5% sodium

dodecyl sulfate, 30 mM sodium pyrophosphate, 5 mM EDTA, 2 mM PMSF, 250 μM leupeptin, 100 μg/mL aprotinin, and 5 mM iodoacetamide. Cell lysates were boiled, passed through a 26 gauge needle, boiled again, and centrifuged.

IX. CN assay.

a) *Peptide labeling.* A synthetic 19 amino acid peptide corresponding to a portion of the regulatory subunit of type II (R_{II}) cAMP-dependent protein kinase (66) (DLDVPIPGRFDRRVSVAAE) was custom ordered from Multiple Peptide Systems (San Diego, CA). A 3.3 mM stock solution (in distilled water) was kept at -20°C. The peptide was labeled in the following phosphorylation buffer: 300 μM ATP, 148.5 μM peptide, 20 mM MES, 200 μM EGTA, 400 μM EDTA, 2 mM $MgCl_2$, 50 μg/mL BSA, 5 μL/mL γ-^{32}P-ATP (10 mCi/mL, 3000 Ci/mmol), and 12 μL/mL protein kinase A (Roche Molecular Biochemicals, Laval, PQ). Following a 60 min incubation at 30°C, the reaction mixture was loaded into a C18 extraction cartridge. The column was washed with 75 mL of 0.1% trifluoroacetic acid after the reaction mixture flowed through. The labeled peptide was then eluted from the column with 2.5 mL 30% acetonitrile in 0.1% TFA. The solvent was evaporated off and the peptide was resuspended in final

peptide buffer (20 mM Tris, pH 8, 100 mM NaCl, 6 mM $MgCl_2$, 100 μM $CaCl_2$, 500 μM DTT, 100 μg/mL BSA).

b) *Phosphatase assay.* The reaction mixture contained 30 μL of extract and 60 μL of assay buffer (20 mM Tris (pH 8), 100 mM NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM DTT, 500 nM okadaic acid, 0.1 mg/ml BSA, 15 μM substrate). Samples were assayed for the ability to dephosphorylate the serine-phosphorylated R_{II} peptide (see previous section). Okadaic acid was added to inhibit PP1 and PP2A activity. Following a 15 min incubation at 30°C, the reaction was stopped with the addition of cold stop solution (100 mMKH_2PO_4, 5% w/v trichloroacetic acid). The hydrolyzed inorganic phosphate was then separated on a Dowex column (Sigma Chemical Co., Oakville, ON) and the columns were washed with a total of 0.9 mL of distilled water. Scintillation fluor (5.5 mL, Canberra Packard Canada Ltd, Mississauga, ON) was added and samples were counted in a scintillation counter. Activity due to protein phosphatase 2C (PP2C), measured as ^{32}P released in assay buffer containing EGTA (200 μM) and in the absence of added calcium, was subtracted from total phosphatase activity (CN+PP2C) to give CN phosphatase activity.

c) *In assay IC50.* Extracts were pooled and 30 μL was added to 10 μL of CsA or FK in assay buffer without peptide. The reaction was started with the addition of 50 μL of assay buffer. Peptide concentration in the assay buffer was adjusted to give a final concentration of 15 μM in the reaction.

d) *Exogenous immunophilin.* Recombinant CyPA (Sigma Chemical Co., Oakville, ON) or FKBP12 (Sigma Chemical Co., Oakville, ON) was added to the assay buffer for a final concentration of 1000 nM in the assay.

X. Protein assay. Protein concentrations of tissue extracts were determined using the BioRad protein assay (BioRad, Hercules, CA) using BSA as the protein standard. Three dilutions were assayed and the average was calculated. The absorbance was read at 690 nm on the EAR 400 AT (SLT-Labinstruments, Austria).

XI. Western analysis. Extracted proteins were separated by SDS-PAGE followed by electrophoretic transfer to nitrocellulose membranes. NFATC2 was detected using a rabbit polyclonal antibody against mouse NFATC2 (Upstate Biotechnology Inc., Lake Placid, NY) followed by horseradish peroxidase (HRP)-conjugated goat affinity

purified $F(ab')_2$ against rabbit Ig (Organon Teknika Corp., Durham, NC), and enhanced chemiluminescence substrate (Amersham Corp., Arlington Heights, IL). Densitometric analysis was used to quantitate bands of interest. CyPA and FKBP12 were similarly detected using polyclonal rabbit antibodies (Affinity Bioreagents, Golden, CO).

XII. Enzyme linked immunosorbent assay (ELISA).

a) *Human IFN-γ.* Tissue culture supernatants were collected from 10 min spins at 4°C, snap frozen, and stored at -20°C. IFN-γ levels were then measured by the Cytokine Laboratory (University of Alberta, Edmonton, Canada).

b) *Mouse IFN-γ.* Tissue culture supernatants were collected from 10 min spins at 4°C, snap frozen, and stored at -20°C. IFN-γ concentration was measured using the Quantikine kit (R&D Systems, Minneapolis, MN) for mouse IFN-γ. Samples that measured outside of the standard curve were diluted with the dilution buffer and re-assayed.

c) *Human and mouse TGF-β1.* Tissue culture supernatants were collected from 10 min spins at 4°C, snap frozen, and stored at -20°C. TGF-β1 levels were measured using the Quantikine kit (R&D Systems, Minneapolis, MN) for TGF-β1. The assay recognizes the active but not

the latent form of TGF-β1. To measure already active TGF-β1, samples were measured without activation. To measure total TGF-β1 (latent and active), samples were activated with HCl for 10 minutes and neutralized with NaOH prior to the assay. Latent TGF-β1 was calculated as the difference between total (acid-activated) and active (not acid-activated). Samples that measured outside of the standard curve were diluted with the dilution buffer and re-assayed.

For analysis of TGF-β1 in patients, whole blood was collected from patients in tubes with EDTA as the anticoagulant and centrifuged at 2500 rpm at room temperature. The plasma layer was transferred to 1 mL Eppendorf tubes and centrifuged at 13000 rpm for 10 minutes at 4°C. These supernatants were then snap frozen and stored at -70°C. With a few exceptions, plasma samples were processed from whole blood within 30 min of collection. As above, active and latent TGF-β1 was measured using the Quantikine kit (R&D Systems, Minneapolis, MN).

d) *Platelet Factor (PF)- 4.* Levels of PF-4 in patient plasma samples were measured using an ELISA kit (Diagnostica Stago, Asnieres, France). Samples were diluted 1/5 but were not activated.

XIII. Immunohistochemistry.

Tissues were harvested, embedded in OCT, snap frozen, and stored at -70°C. Sections (0.4 μm) were cut onto poly-L-lysine coated microscope slides by the laboratory technician, Ms. Joan Urmson, and stored at -20°C. Tissue sections were briefly brought to room temperature and fixed with 4% paraformaldehyde for one hour. Slides were then washed in PBS (3 x 15 min) and endogeneous peroxidase activity was quenched using 1% hydrogen peroxide in PBS (2 x 2 min then 1 x 10 min). Slides were then rinsed briefly and blocked with 20% normal secondary antibody serum for at least 2 hours at room temperature. Slides were then briefly rinsed and endogenous avidin/biotin sites were blocked using the blocking kit (Zymed Laboratories, Inc, San Francisco, CA). Primary antibody incubation was overnight at 4°C in a humidified chamber. Secondary antibody was applied at room temperature for 30 min after three 15 min washes in PBS. Following another three 15 min washes in PBS, sections were incubated with the avidin-biotin complex (Vector Laboratories, Inc., Burlingame, CA) at room temperature for 30 min. After another three rounds of PBS, 3,3'-diaminobenzidine substrate (Sigma Chemical Co., Oakville, ON) was added to the sections and the color was allowed to develop for 5-10 min. Slides were then rinsed 3X with distilled water and counterstained with Alcian blue for 10 min. Following a brief rinse

in distilled water, slides were dehydrated with 5X ethanol and 3X xylene. Slides were mounted with Permount (Fischer Scientific, Fair Lawn, NJ). Pictures were taken with the Olympus BX40 microscope and Spot camera (Diagnostic Instruments, Inc.).

XIV. RNA extraction

Total RNA was extracted from tissue from individual mice using a modified method described by Chirgwin (260). Tissues were homogenized in 2.5 mL of 4 M guanidium isothiocyanate and spun at 10,000 rpm (Sorvall, RC-5B) for 10 min at 4°C. Supernatants were then layered on 0.9 mL $CsCl_2$ and centrifuged for 4-5 hours at 15°C and 45,000 rpm. RNA concentrations were determined by absorbance at 260/280 nm. The quality of the RNA was determined by Northern analysis, performed by Mr. Luis Guembes Hidalgo.

XV. RNAse protection assay.

TGF-β1 mRNA levels were analyzed by RNase protection assay using the Riboquant multiprobe set (BD PharMingen, San Diego, CA) following the instructions of the supplier. In brief, 5 μg of RNA obtained from kidneys of individual mice were hybridized overnight to the ^{32}P-labeled anti-sense RNA probe, which had been previously synthesized from the supplied DNA template set (mCK-3 from PharMingen).

Single-stranded RNA and free probe were digested by RNase A and T1. Protected RNA was phenolized, precipitated, and resolved on a 5% denaturing polyacrylamide gel. Kodak X-Omat AR film was exposed to the gel for 15 minutes with two intensifying screens at -70°C. The quantity of protected RNAs was determined using a PhosphorImager. TGF-β1 transcripts were identified by the length of the respective fragments. For quantitation, TGF-β1 values were expressed as a percentage of the values of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each gel lane. Two of three mice were analyzed per group and averaged.

XVI. Real time PCR (Taqman)

Total RNA (1 μg) was reverse transcribed by Maloney murine leukemia virus transcriptase in a final volume of 40 μL. For detection of mouse TGF-β1 and β-actin, primers were obtained from the University of Alberta. Probes were purchased from Biosource International (Camarillo, CA). All primers and probes were designed by Dr. Birgit Sawitzki (Institute of Medical Immunology, Humboldt University, Berlin, Germany) and are as follows:

TGF-β1

Forward primer: 5' GGC TAC CAT GCC AAC TTC TGT CT 3'

Reverse primer: 5' CCG GGT TGT GTT GGT TGT AGA 3'

Probe: 5'– FAM-CACACAGTACAGCAAGGTCCTTGCCCT-TAMRA-3'

β-actin

Forward primer: 5' CGG TTC CGA TGC CCT GA 3'

Reverse primer: 5' CGG ATG TCA ACG TCA CAC TTC A 3'

Probe: 5'-FAM-CAGCCTTCCTTCTTGGGTATGGAATCCT-TAMRA-3'

All PCRs were performed in duplicate from 0.5 μL cDNA per 25 μL reaction volume on an ABI Prism 7700 Sequence Detection System (Taqman).

Chapter Three

Comparing CsA and FK: inhibition of CN phosphatase activity, NFATC2 dephosphorylation, and IFN - γ secretion

Versions of this chapter have been accepted for publication.

Batiuk, T.D., **Kung, L.**, and Halloran, P.F. Evidence that calcineurin is rate-limiting for primary human lymphocyte activation. Journal of Clinical Investigation 100:1894-1901, 1997.

Kung, L. and Halloran, P.F. Immunophilins may limit calcineurin inhibition by cyclosporine and tacrolimus at high drug concentrations. Transplantation 70(2):327-35, 2000.

Although CsA and FK are qualitatively similar in their action on CN and events downstream of CN, these drugs are quantitatively different. I hypothesized that differential inhibition of CN was one molecular basis for the greater molar potency of FK versus CsA. To test this hypothesis, I compared their 50% inhibiting concentrations for CN phosphatase activity and two events downstream of CN.

I. Inhibition of NFATC2 dephosphorylation and IFN-γ secretion

NFATC2 dephosphorylation and activation

The first step was to set up the Western blot system for NFATC2 in normal cells and to demonstrate the dephosphorylation of NFATC2 in immune cells activated in culture. I stimulated mouse T cells with 0.5 μM, 1 μM, 2 μM or 10 μM ionomycin for 5 min and examined NFATC2 dephosphorylation by Western analysis on a 6% SDS-PAGE gel. NFATC2 in unstimulated cells ran as one distinct band at ~140 kD. Following stimulation by ionomycin, NFATC2 was serine-dephosphorylated by CN and there was a downward shift of the band to ~120 kD (Figure 7). The band also became more diffuse and this was likely a result of the different phosphorylation states of NFATC2. I also explored the activation of NFATC1, another member of the NFATC family. Three distinct bands in unstimulated cells

became smeared following ionomycin stimulation (Figure 8). The detection of multiple bands by this antibody may represent cross-reactivity with other NFATC family members or may reflect the greater number of isoforms for NFATC1. I also examined the time course for NFATC2 dephosphorylation following ionomycin stimulation of mouse spleen cell suspensions. SCS were stimulated with 2 μM of ionomycin and harvested at 1, 2, and 4 hours. I found that the dephosphorylation of NFATC2 remained stable over 4 hours (Figure 9).

The stimulation of cells with a calcium ionophore is unphysiologic. Consequently, I explored the effect of anti-CD3 antibody - a more physiologic stimulus - on NFATC2 in mouse spleen cells. SCS were stimulated with 0.5 μg/mL, 1.5 μg/mL, or 4.5 μg/mL of anti-CD3 antibody for 1 hour and NFATC2 was analyzed in whole cell extracts. Ionomycin-stimulated SCS were included for comparison. I found that the effects of anti-CD3 stimulation were comparable to those with ionomycin (Figure 10). Subsequent examination of the time course (0.5, 1, 2, 4 h) for stimulation of spleen cell suspensions by 1.5 μg/mL anti-CD3 showed that the activation/dephosphorylation of NFATC2 was also stable over 4 hours (Figure 11).

Inhibition of NFATC2 dephosphorylation by CsA and FK

To determine the effect of CsA on NFATC2 dephosphorylation, I treated enriched mouse T cells with 0, 1, 10, 100, or 1000 ng/mL CsA for 30 min and then stimulated with 2μM of ionomycin for 5 min. As expected, increasing concentrations of CsA inhibited the dephosphorylation of NFATC2 and subsequently the downward shift of the band (Figure 12). Inhibition of NFATC2 dephosphorylation by CsA was complete and the IC50 was 17.6 ng/mL (95% CI 4-68 ng/mL). I then examined the effect of CsA on mouse SCS under the same conditions. The same pattern of expression was observed, with an IC50 of 7.1 ng/mL (Figure 13). The IC50 for CsA in human T cells was previously determined to be 20 ng/mL (95% CI 15-26 ng/mL) (261).

I also examined the effects of FK on NFATC2 in mouse T cells, comparing them to CsA. Enriched populations of mouse T cells were treated with 0, 0.01, 0.1, 1, 10, or 100 ng/mL FK for 30 minutes and then stimulated with 2 μM ionomycin for 5 min. NFATC2 in whole cell extracts was analyzed by Western blot. I found that FK inhibited NFATC2 dephosphorylation in a manner similar to CsA, except that the IC50 was lower (0.7 ng/mL, 95% CI 0.2-2.2 ng/mL) (Figure 14). I also looked at the effect of FK on NFATC2 in human T cells under the same conditions and found a similar pattern, with an IC50 of 0.1 ng/mL (Figure 15). Thus both CsA and FK completely inhibit NFATC2

dephosphorylation in a concentration-dependent manner; however, the IC50s for FK were approximately 24- to 70-fold lower than those for CsA.

Inhibition of IFN-γ

Next I explored the inhibition of IFN-γ secretion by FK in human PBL and mouse spleen cell suspensions in culture. PBL or spleen cell suspensions were treated with increasing concentrations of FK for 30 minutes prior to stimulation with 5 μM ionomycin for 4 hours. Cultures were then spun down and the supernatant collected for analysis of IFN-γ by ELISA. Inhibition of IFN-γ secretion by FK was complete, with an IC50 of 0.22 ng/mL (95% CI 0.14-0.34 ng/mL) in human PBL and 0.1 ng/mL (95% CI 0.04-1.4 ng/mL) in mouse spleen cell suspensions (Figure 16). The IC50s for FK were approximately 32- and 60-fold lower than those for CsA (261).

II. Inhibition of CN Activity

I then examined the ability of FK to inhibit CN phosphatase activity in mouse spleen cells and human PBL in culture. SCS or PBL were treated for 30 minutes with FK and CN inhibition was assessed in whole cell extracts. Similar to NFATC2 dephosphorylation and IFN-γ secretion, FK inhibited CN phosphatase activity in a concentration

dependent manner (Figure 17). The IC50 for FK in PBL was 0.23 ng/mL (95% CI 0.07 – 0.75 ng/mL), which was 43-fold higher than that for CsA (262). Under these conditions, SCS were minimally inhibited by FK and an IC50 could not be reasonably determined (Figure 18).

In contrast to the two downstream events, the inhibition of CN activity was incomplete at saturating concentrations of FK. To determine whether this was also the case in other cell types, I assessed the inhibition of CN by FK and CsA in human whole blood and mouse whole blood. Whole blood was collected from healthy volunteers by venous puncture or from mice by cardiac puncture. Whole blood was treated with 500 ng/mL of FK or 1000 ng/mL of CsA for 30 minutes at 37°C and whole cell extracts were analyzed for CN activity. As in human PBL and mouse SCS, CN phosphatase activity was incompletely inhibited by FK and CsA in human and mouse whole blood. Furthermore, with the exception of human whole blood, FK generally inhibited less CN phosphatase activity than CsA. Thus incomplete CN inhibition was not cell- or species-specific and occurred with both drugs (Table 2, Chapter 4). The incomplete inhibition of CN activity by CsA and FK is examined in greater detail in the next chapter.

FK inhibition of CN in human whole blood

This laboratory has previously shown that CN activity is only partially inhibited in transplant patients on CsA therapy (263;264). I subsequently examined the effect of FK on CN activity in patients just starting FK-based immunosuppression. First, to establish an *in vitro* frame of reference, I determined the IC50 of FK in human whole blood. I treated 0.5 mL of human whole blood with increasing concentrations of FK for 30 min at 37°C and assessed CN activity in whole cell lysates. The *in vitro* IC50 of FK in human whole blood was 12.7 ng/mL (n=5, 95% CI 8.9 – 18.1 ng/mL) and the maximum CN inhibition averaged 75%, ranging from 73-79% (Figure 19). To evaluate the effect of FK on CN *in vivo*, CN activity was assessed in whole blood from transplant patients before and after starting FK-therapy. Samples were collected pre-FK, 1 hour post-FK (peak), and 12 hour post-FK (trough). In both patients, CN activity was inhibited approximately 40% at peak (39.4 ng/mL) and at trough (12.5 ng/mL, 14 ng/mL) (Figure 20).

Inhibition of CN by FK in vivo in mice

This laboratory has previously shown the effects of CsA *in vivo*. Blood and tissue levels of CsA peaked at 1 h, concomitant with the

greatest inhibition of CN activity. In addition, the accumulation of CsA and the inhibition of CN activity was greatest in the kidney and liver (Kung *et al* submitted). There have not been any *in vivo* studies of FK examining the relationship between FK levels and CN inhibition. I hypothesized that the dynamics and kinetics of FK would be similar to that of CsA.

BALB/c mice were fed by oral gavage 10 mg/kg of FK dissolved in PEG400. Tissues were then harvested at 0 h, 0.5 h, 1 h, 2 h, 4 h, 24 h, 48 h, and 72 h. Tissues from four mice were pooled for analyses of CN activity and FK levels. CN activity was calculated as the percentage of CN activity at 0 h and levels of FK were expressed as nanograms of FK per gram of wet weight tissue. FK levels were determined by Isotechnika using an ELISA-based assay. Due to the high cost of determining FK levels, only whole blood, spleen, and kidney were examined. Similar to CsA, levels of FK peaked at 1 h and were significantly higher in kidney compared to spleen or whole blood (Figure 21). The inhibition of CN also correlated with FK levels. It is not clear why there were still significant levels of FK in the kidney at 48 h and 72 h, particularly since CN activity had essentially recovered at these time points.

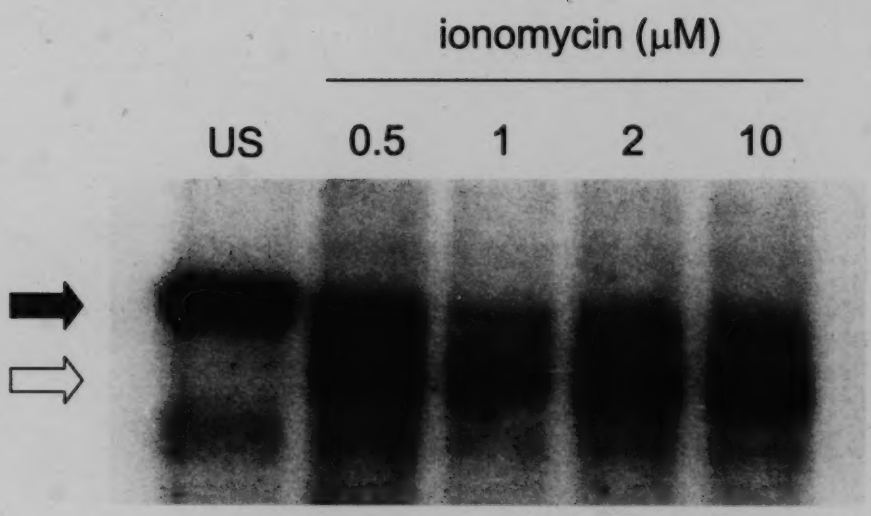


Figure 7. NFATC2 dephosphorylation in mouse T cells following ionomycin treatment. Western blot of NFATC2 enriched populations of mouse T cells unstimulated or stimulated with 0.5 μM, 1 μM, 2 μM, or 10 μM ionomycin for 5 minutes. Whole cell extracts were run on a 6% SDS-PAGE, electrotransferred to nitrocellose membrane and immunoblotted with anti-NFATC2 antibody. In unstimulated (US) cells, NFATC2 is phosphorylated and runs at ~140 kD (black arrow). Following treatment with ionomycin, NFATC2 is dephosphorylated NFATC2 and runs at ~120 kD (white arrow). This experiment was performed once.

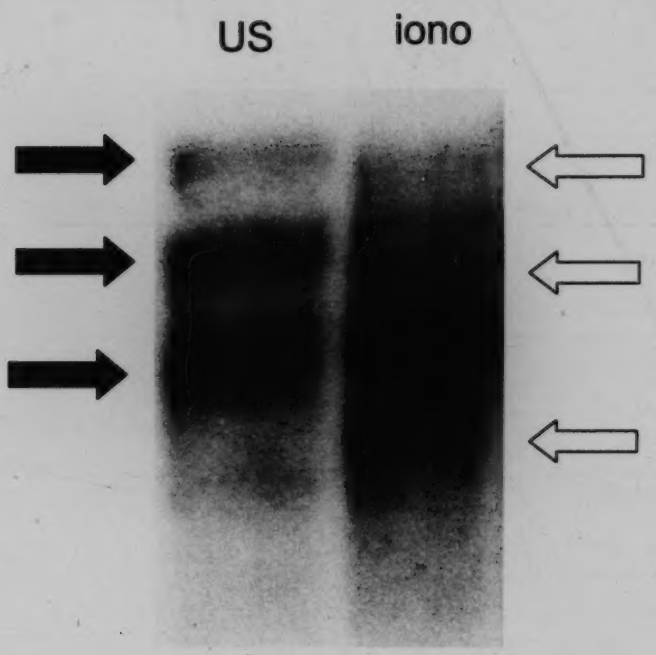


Figure 8. Western of NFATC1 in mouse spleen cells following ionomycin stimulation. NFATC1 in unstimulated (US) spleen cells is detected as three distinct bands (black arrows). Following stimulation with 2 μM ionomycin for 5 minutes (iono), NFATC1 is dephosphorylated and there is a downward smear of the bands (white arrows). This experiment was performed once.

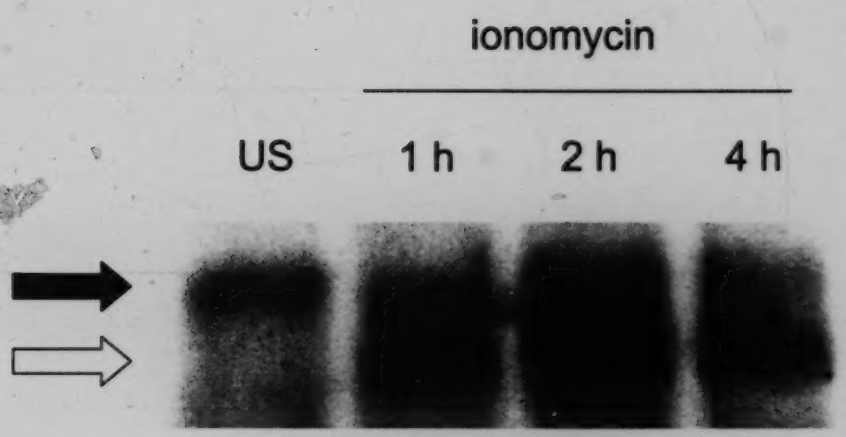


Figure 9. Time course for ionomycin stimulation of spleen cell suspensions. Mouse spleen cell suspensions were stimulated with 2 μM of ionomycin for 1, 2, or 4 hours. Whole cell extracts were run on a 6% SDS-PAGE, electrotransferred to nitrocellose membrane and immunoblotted with anti-NFATC2 antibody. The shift of NFATC2 band from ~140 kD (black arrow) in unstimulated cells to ~120 kD (white arrow) in stimulated cells is stable for at least 4 hours. This experiment was performed once.

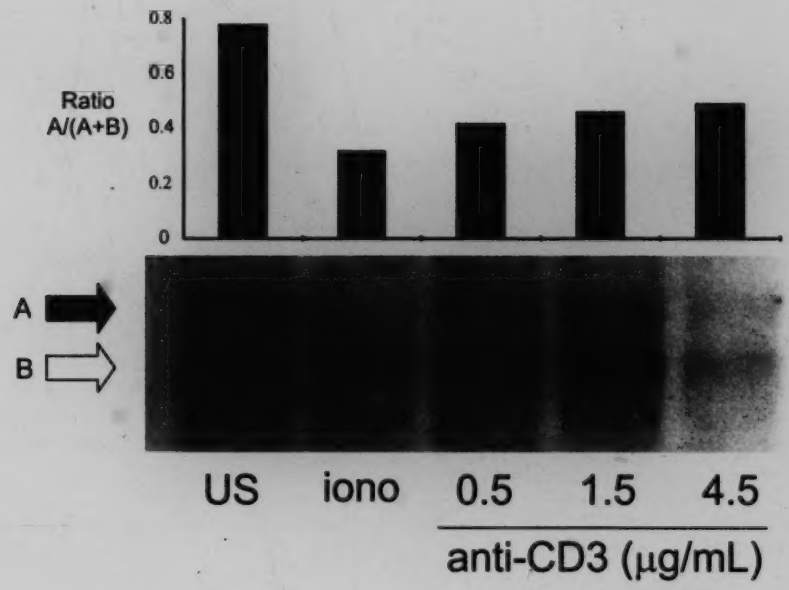


Figure 10. NFATC2 dephosphorylation following stimulation of spleen cell suspensions by anti-CD3 antibody. Spleen cells were stimulated with 1.5, 1.5, or 4.5 μg/mL of anti-CD3 antibody for 1 hour and NFATC2 was analyzed in whole cell extracts on a 6% SDS-PAGE. Ionomycin (2 μM) stimulation was included as a positive control for stimulation. Graph above blot depicts densitometric analysis of NFATC2: the Y-axis represents the value of the upper band (black arrow, A) over the total value of the upper (black arrow, A) and lower (white arrow, B) bands. This experiment was performed once.

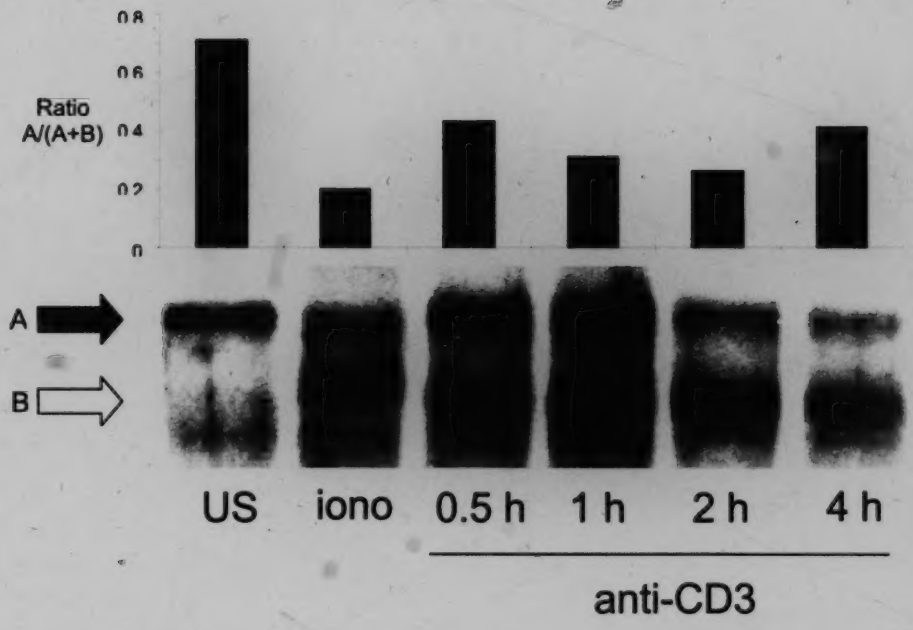


Figure 11. Time course for NFATC2 dephosphorylation by anti-CD3 antibody in spleen cells. Spleen cells were stimulated with 1.5 μg/mL of anti-CD3 antibody for 1, 2, or 4 hours and NFATC2 was analyzed in whole cell extracts on a 6% SDS-PAGE. Ionomycin (2 μM, 4 h) stimulation was included as a positive control for stimulation. Graph above blot depicts densitometric analysis of NFATC2: the Y-axis represents the value of the upper band (black arrow, A) over the total value of the upper (black arrow, A) and lower (white arrow, A) bands. This experiment was performed once.

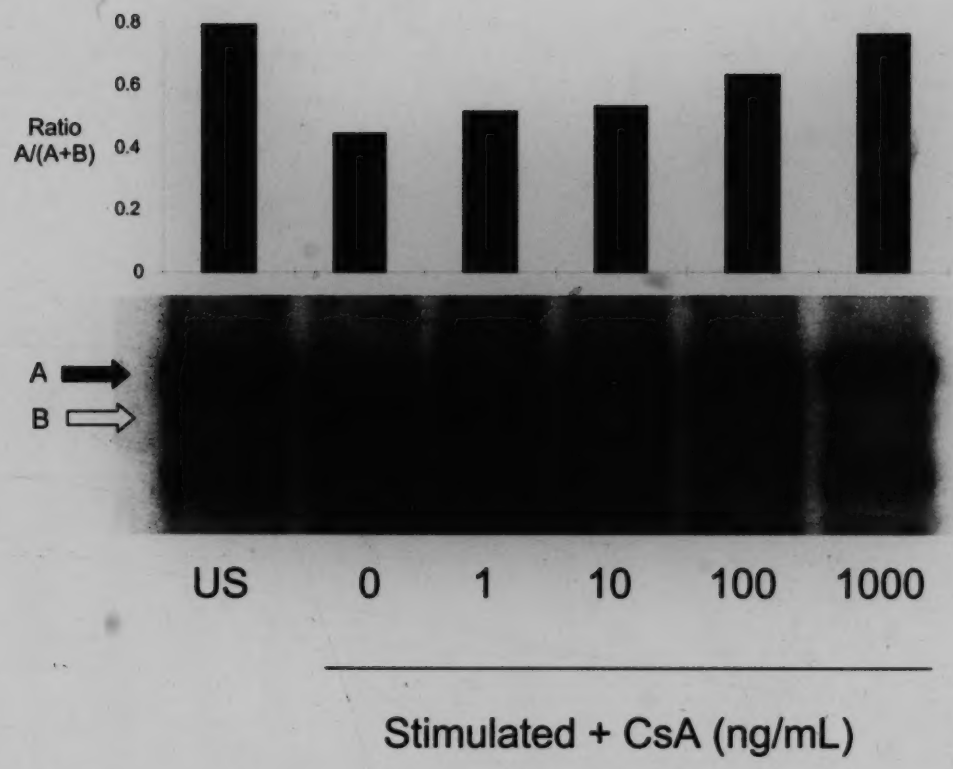


Figure 12. CsA inhibition of NFATC2 dephosphosphorylation in ionomycin-stimulated mouse T cells. Enriched mouse T cell populations were treated for 30 minutes with CsA followed by stimulation with ionomycin (2 μM) for 5 minutes. Unstimulated cells not treated with CsA were also included as a control. NFATC2 was analyzed in whole cell extracts on a 6% SDS-PAGE. Graph above blot depicts densitometric analysis of NFATC2: the Y-axis represents the value of the upper band (black arrow, A) over the total value of the upper (black arrow, A) and lower (white arrow, B) bands. This experiment is representative of two independent experiments.

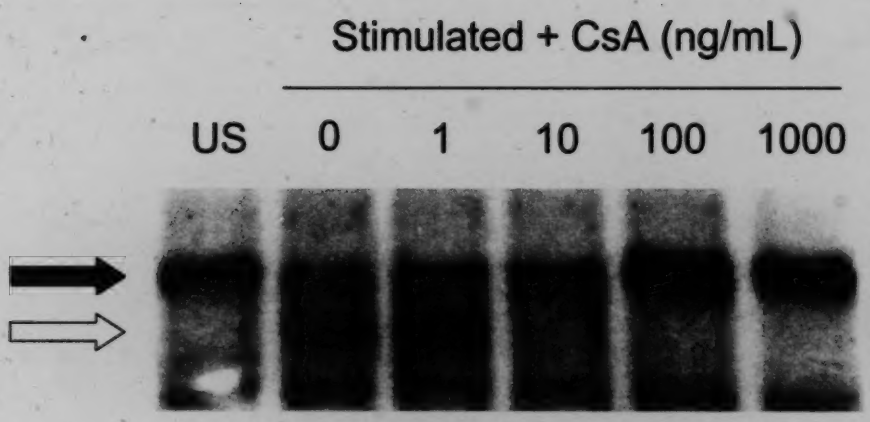


Figure 13. Inhibition of NFATC2 dephosphosphorylation by CsA in ionomycin-stimulated mouse spleen cell suspensions. Spleen cell suspensions were treated for 30 minutes with CsA followed by stimulation with ionomycin (2 μM) for 5 minutes. Unstimulated cells not treated with CsA were also included as a control. NFATC2 was analyzed in whole cell extracts on a 6% SDS-PAGE. Densitometric analysis of NFATC2 (the upper band (black arrow) over the total of the upper (black arrow) and lower (white arrow) bands) was done to calculate the IC50. This experiment is representative of two independent experiments.

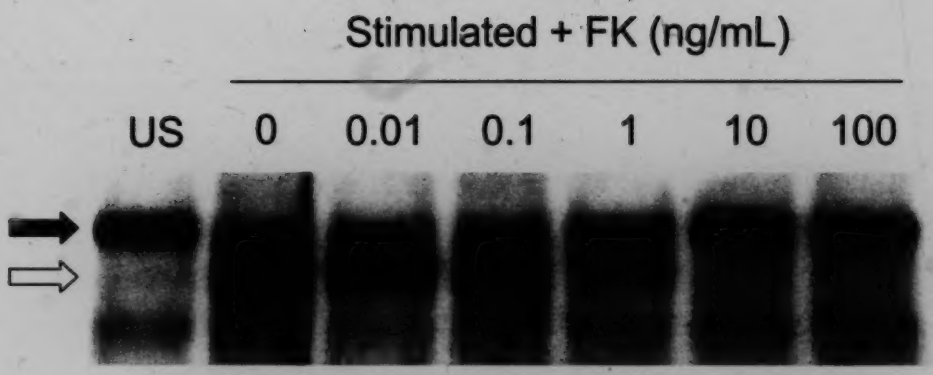


Figure 14. Inhibition of NFATC2 dephosphosphorylation by FK in ionomycin-stimulated mouse T cells. Enriched mouse T cell populations were treated for 30 minutes with FK followed by stimulation with ionomycin (2 μM) for 5 minutes. Unstimulated cells not treated with FK were also included as a control. NFATC2 was analyzed in whole cell extracts on a 6% SDS-PAGE. Densitometric analysis of NFATC2 (the upper band (black arrow) over the total of the upper (black arrow) and lower (white arrow) bands) was done to calculate the IC50. This experiment is representative of two independent experiments.

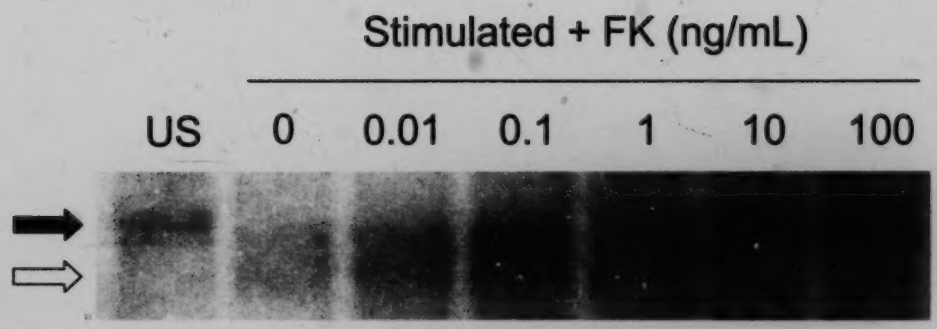
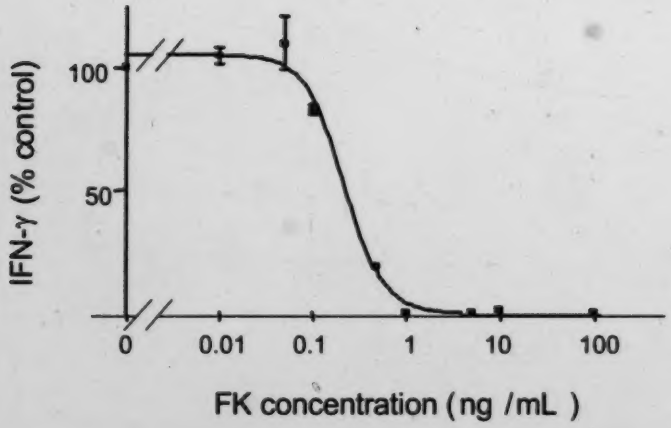


Figure 15. FK inhibits NFATC2 dephosphosphorylation in human T cells stimulated with ionomycin. T cells were treated for 30 minutes with FK followed by stimulation with ionomycin (2 μM) for 5 minutes. Unstimulated cells not treated with FK were also included as a control. NFATC2 was analyzed in whole cell extracts on a 6% SDS-PAGE. Densitometric analysis of NFATC2 (the upper band (black arrow) over the total of the upper (black arrow) and lower (white arrow) bands) was done to calculate the IC50. This experiment is representative of two independent experiments.

A.



B.

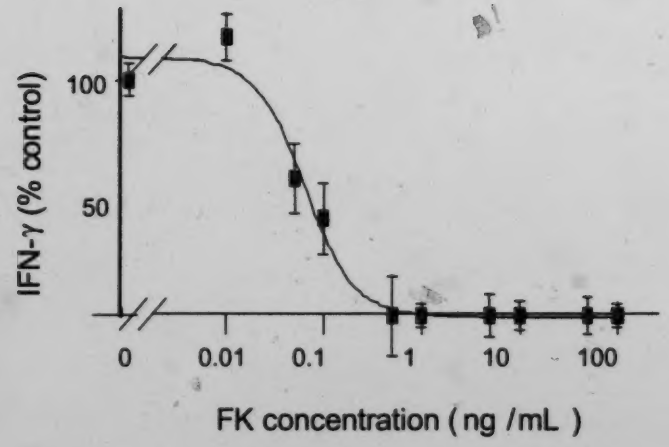


Figure 16. FK inhibits IFN-γ secretion. Human PBL (a) or mouse spleen cells (b) were treated for 30 minutes with FK followed by stimulation with ionomycin (5 μM) for 4 hours at 37°C. IFN-γ concentrations in the supernatant were determined by ELISA and plotted against the FK concentration on GraphPad Prism. These experiments are representative of two independent experiments.

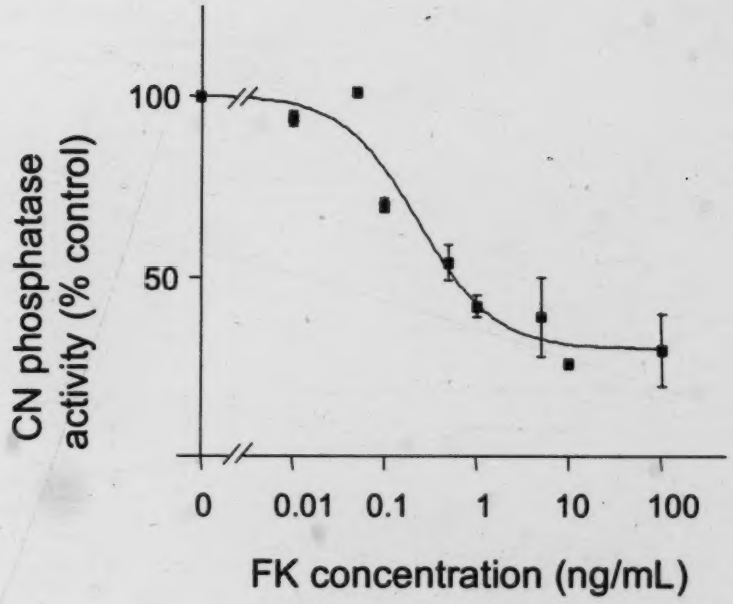


Figure 17. FK inhibits CN phosphatase activity in a concentration-dependent manner in human PBL. Human PBL were treated for 30 minutes with FK followed at 37°C. CN phosphatase activity was determined against a synthetic 19 amino acid peptide and plotted against the FK concentration. This graph represents four independent IC50 determinations.

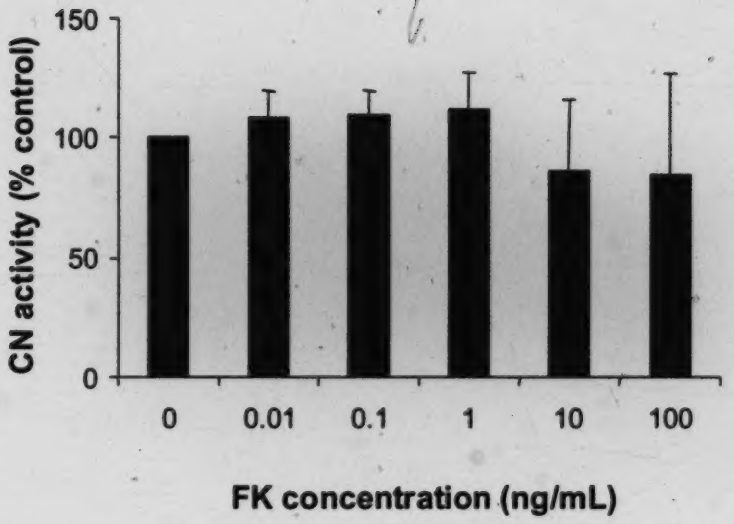


Figure 18. FK minimally inhibits CN phosphatase activity in mouse spleen cells. Spleen cells were treated for 30-60 minutes with FK at 37°C. CN phosphatase activity was determined against a synthetic 19 amino acid peptide and plotted against the FK concentration. This graph represents three independent IC50 determinations.

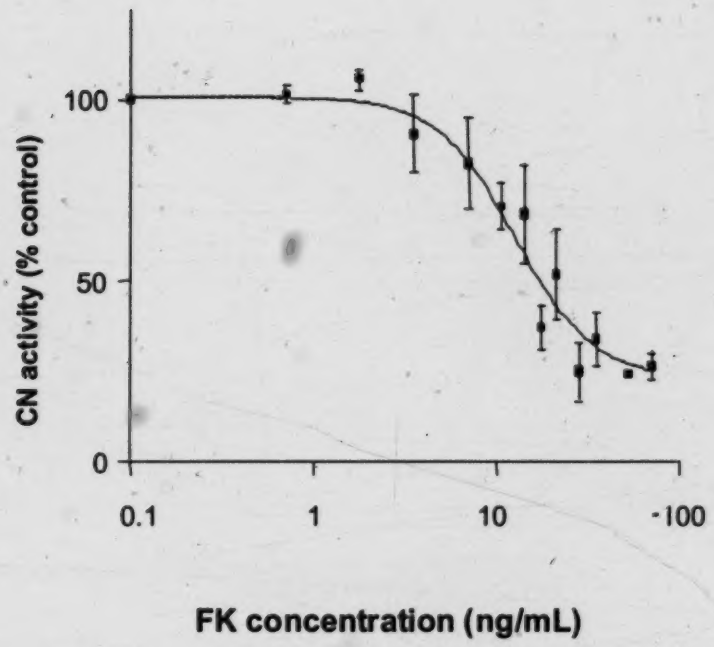


Figure 19. FK inhibits CN phosphatase activity in human whole blood *in vitro*. Whole blood was treated for 30 minutes with FK at 37°C. CN phosphatase activity was determined against a synthetic 19 amino acid peptide and plotted against the FK concentration. Graph depicts the average of five independent experiments. This graph represents six independent IC50 determinations.

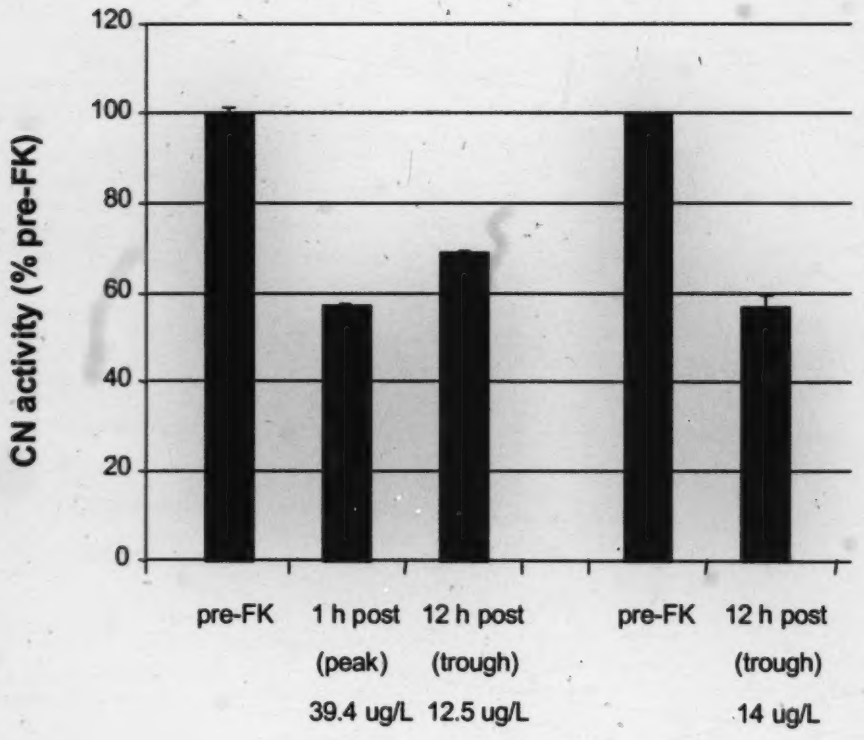


Figure 20. CN activity is inhibited in transplant patients following commencement of FK-therapy. Whole blood was collected before the first dose of FK, after 1 h post-dose (peak), and after 12 h post-dose (trough). Two patients were examined and CN activity was expressed as a percentage of CN activity before the first FK dose.

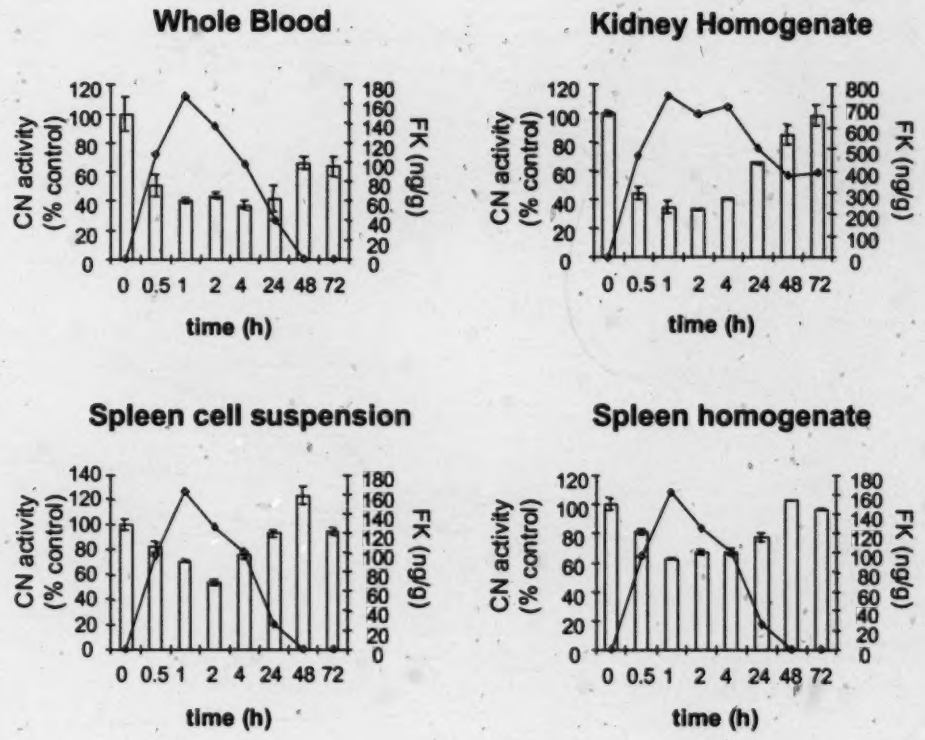


Figure 21. Inhibition of CN by FK *in vivo* in mice. Mice were fed 10 mg/kg of FK by oral gavage and tissues were harvested at 0 h, 0.5 h, 1 h, 2 h, 4 h, 24 h, 48 h, and 72 h. Tissues were homogenized in lysis buffer for the CN assay or in PBS for determination of drug levels. Spleen cell suspensions were lysed in lysis buffer. CN activity (bars) was calculated as a percentage of the activity at 0 h. The amount of FK (lines) in tissue homogenates was determined and expressed as nanograms per gram of tissue. There were 4 mice per time point and tissues were pooled for analyses.

Chapter Four

Exploring the basis for the incomplete inhibition of CN phosphatase activity by CsA and FK

A version of this chapter has been accepted for publication.

Kung, L. and Halloran, P.F. Immunophilins may limit calcineurin inhibition by cyclosporine and tacrolimus at high drug concentrations. Transplantation 70(2):327-35, 2000.

A version of this chapter has been submitted for publication.

Kung, L., Batiuk, T.D., Noujaim, J., Palomo, S., Helms, L.M., and Halloran, P.F. Tissue distribution of calcineurin and its sensitivity to inhibition by cyclosporine. American Journal of Transplantation.

In chapter three, I made the unexpected observation that CN activity could not be completely inhibited in the assay by CsA and FK in particular tissues. This was in contrast to the complete inhibition of events downstream of CN, namely NFATC2 dephosphorylation and IFN-γ secretion. I explored a number of possible bases for the incomplete CN inhibition in the assay, beginning with some of the technical aspects of the assay.

I. Determining the basis for incomplete CN inhibition

The drug-immunophilin complexes are not competitive inhibitors of CN; they do not inhibit the dephosphorylation of small peptide substrates and even enhance the activity of CN against certain substrates, namely PNPP. Subsequently, I determined whether the incomplete CN inhibition in the assay was substrate dependent. I compared the inhibition of CN phosphatase activity against the R_{II} peptide with that against an unrelated 31 amino acid substrate derived from an analogue of protein phosphatase inhibitor peptide (a generous gift from Dr. C. Holmes, University of Alberta, Edmonton, Canada). Human PBL were treated in culture with 0, 0.1, 1, 10, or 100 ng/mL FK and whole cell extracts were assayed for CN activity against the serine-phosphorylated 31-mer peptide. As with the 19-mer, inhibition

of CN activity by FK was incomplete (Figure 22). Thus the lack of complete inhibition by FK was not specific for the R_{II} peptide.

I then tested the specificity of the assay system. Rapamycin is an immunosuppressive drug that binds FKBP but does not inhibit CN. SDZ (kindly provided by Novartis Canada) is a CsA analogue that binds CyP but does not inhibit CN. Thus as competitors for FKBP and CyP, rapamycin and SDZ specifically reverse the inhibition of CN phosphatase activity by FK and CsA, respectively, and can be used to confirm the specificity of the assay system. PBL were treated with 0, 0.1, 1, 10, or 100 ng/mL FK for 30 min at 37°C, after which rapamycin (1000 ng/mL) or SDZ (100000 ng/mL) was added to the culture for another 30 min. Whole cell extracts were analyzed for CN activity. Rapamycin but not SDZ specifically reversed the inhibition of CN by FK, confirming the specificity of this assay system (Figure 23). Reversal occurred whether rapamycin was added to the culture before or after FK (Figure 24). Interestingly, I found that rapamycin could reverse FK inhibition of CN phosphatase if added in culture but not if added to the assay (Figure 25). This laboratory has also shown that SDZ reverses CsA but not FK inhibition of CN when added in culture or in assay (Noujaim and Halloran, unpublished results).

I hypothesized that more time may be required for the drug-immunophilin-CN complexes to reach equilibrium in the assay. To test

this hypothesis, I incubated lysates from untreated cells with 500 ng/mL FK for 5 minutes at 37°C prior to addition of the peptide substrate. CN activity in these lysates was compared to that in lysates that were assayed without the pre-incubation. I found that the pre-incubation did not result in complete inhibition of CN activity by FK in the assay (Figure 26). Since the assay was initially demonstrated with Jurkat T cells, I assessed the FK inhibition of CN activity in these cells. I found that the inhibition of CN by FK in these cells was essentially complete (Figure 27).

To determine whether the incomplete inhibition of CN by FK was a result of in culture treatment, I fed mice with 0, 10, or 25 mg/kg of FK and assayed the CN activity in whole blood, spleen cell suspensions, spleen homogenates, and kidney homogenates at 1 hour post dose (when FK levels in the blood peak). I found that FK inhibition of CN was still incomplete and this was not a result of loss of FK during the extraction process, since additional FK added directly to the assay did not increase CN inhibition (Figure 28). Thus the lack of complete inhibition of CN by FK was not due to in culture treatment of cells.

I hypothesized that immunophilins are limiting in the assay. To test this hypothesis, I added increasing concentrations of recombinant FKBP12 to the assay buffer. The addition of 1000 nM FKBP12 to the

assay buffer along with 500 ng/mL of FK resulted in near complete inhibition of CN phosphatase activity in human PBL, mouse spleen cell suspension, mouse whole blood, and mouse brain homogenate. (Table 3). Furthermore, the effect of FKBP12 was concentration dependent (Figure 29). CN inhibition by CsA was incomplete in mouse heart and brain. I subsequently examined whether CsA inhibition of CN could be increased with exogenous CyPA in mouse brain and heart homogenates. Similar to FKBP12, CyPA (1000 nM) increased the CN inhibition by CsA (1000 ng/mL) in both tissues (Figure 30). These findings were surprising since immunophilins have been long thought to not be limiting for CsA and FK inhibition of CN.

To determine whether immunophilins might be lost during the extraction process and thus become limiting in the assay, I compared CN inhibition by CsA and FK in entire cell lysates (extract supernatant plus extract pellet) and extract supernatant. The degree of inhibition in the entire lysate was no greater than that in the extract supernatant, suggesting that limiting FKBP12 is not a result of protein loss during the extraction process (Figure 31). However while the physical loss of immunophilins is unlikely, there might still be functional loss.

To determine the FK concentration at which FKBP12 becomes limiting, I assessed inhibition of CN phosphatase activity in PBL treated in culture with 0, 0.1, 1, 10, or 100 ng/mL FK and performed the assay

with or without exogenous FKBP12. I found that exogenous FKBP12 increased inhibition of CN phosphatase activity only at the higher concentrations of FK (Figure 32). Thus at these high drug concentrations, FK is present in the system but is unable to inhibit CN phosphatase activity until sufficient FKBP12 is added.

II. Differential inhibition of CN in different tissues

From the initial survey of CN inhibition by FK in tissues (Table 3), I hypothesized that the degree of maximal CN inhibition by FK may vary among tissues and contribute to the clinical effects of FK. To test this hypothesis, I determined the maximum inhibition of CN by FK (1000 ng/mL) in mouse spleen, kidney, heart, liver, and brain in the absence and in the presence of exogenous FKBP12. I found the greatest degree of inhibition in liver and the lowest degree in the brain. Furthermore, CN inhibition in all tissues could be increased to near completion with exogenous FKBP12 (Table 2). This laboratory has also shown a similar pattern of CN inhibition in mouse tissue homogenates for CsA (Kung *et al* submitted).

Since FKBP12 was limiting only at high FK concentrations in PBL (previous section), I determined whether this was also the case for mouse tissues. CN activity in spleen, kidney, liver, heart, and brain homogenates was assessed over a range of 0 – 1000 ng/mL FK in the

presence or absence of exogenous FKBP12. As in human PBL, I found that FKBP12 was limiting only at high drug concentrations. However, when the IC50s were calculated, I was surprised to find that the IC50s for FK were in the same range as those for CsA (Table 4). This was in contrast to the data presented in chapter two, where the molar potency of FK was 25- to 80-fold greater than CsA in culture in PBL suspensions.

One difference between the experiments in chapter three and those presented in this chapter was that drug exposure occurred either before (chapter three) or after (this chapter) cell disruption; that is, intact cells (chapter three) or cell lysates (this chapter) were treated with drug. It may also be a difference between cells vs. organs. To address these hypotheses, I assessed CN activity in PBL that were either exposed to FK in culture or that were lysed and the extracts were exposed to FK in the assay. Interestingly, maximum inhibition of CN by FK was greater if intact cells were treated than if extracts from disrupted cells were treated. Furthermore, exogenous FKBP12 increased CN inhibition regardless if FK treatment occurred before or after cell lysis (Figure 33). When the IC50s were calculated for intact cells vs. cell lysates, I found a 10-fold difference. If drug exposure occurred prior to cell disruption, the IC50 was 2 ng/mL (95% CI 1-3 ng/mL). If drug exposure occurred after cell disruption, the IC50 was

19 ng/mL (95% CI 12-28 ng/mL). Finding this difference with FK, I explored whether this also occurred with CsA and repeated the experiment with CsA. Interestingly, I found that the curves for CsA over-lapped (Figure 33). That is, the IC50 of CsA was the same regardless of whether drug exposure was pre- (16 ng/mL, 95% CI 12-22 ng/mL) or post-disruption (29 ng/mL, 95% 22-38 ng/mL).

III. Immunophilins in tissues

Our laboratory has proposed the hypothesis that the tissue distribution of CsA and the relative ratio of CN to active CyPs contribute to the clinical effects of the drug (Kung et al, submitted). The abundance of immunophilins in the tissue may be a factor in the accumulation of CsA as well as the maximum degree of CN inhibition by CsA in the tissue. This laboratory and others have shown that CN activity varies among tissues (265;266)(Kung *et al* submitted). CN activity is greatest in the brain (~200 % peptide hydrolyzed/min/mg protein) and ranges from 5 – 25 % peptide hydrolyzed/min/mg protein in the spleen, kidney, heart, and liver. The maximum CN inhibition by CsA and FK varies between the two drugs. CsA inhibits most of the CN in spleen, liver, and kidney (at least 80%); on the other hand, FK inhibits only about half in the same tissues (Table 3). I hypothesized that the maximal degree of CN inhibition by CsA or FK is determined in part by the relative abundance of active immunophilins in the tissue.

That is, the present data would suggest that there is generally more active cyclophilin than active FKBP in the spleen, liver, heart, and kidney. To test this hypothesis, I examined the relative abundance of CyPA and FKBP12 in spleen, kidney, heart, liver, and brain by Western blot. The wet weights of tissues were determined and tissues were homogenized in RIPA buffer (1 g/3 mL). Except for brain (5 μL), the same volume of tissue extracts (10 μL) was loaded on a 12% SDS-PAGE gel.

The protein expression of FKBP12 and CyPA differed among the tissues examined (Figure 34). With the exception of brain, FKBP12 was barely detectable in spleen, kidney, liver, and heart (less than 0.2 μg/10 μL extract). In contrast, CyPA was greater than 0.2 μg/10 μL extract in all tissues. The finding that the protein expression of FKBP12 was less than that of CyPA is in agreement with the observed differences in maximum CN inhibition by CsA and FK. That is, the lesser degree of maximum CN inhibition by FK compared to CsA had suggested that FKBP12 would be more limiting in the tissues than CyP. However, I only examined FKBP12 and CyPA. Although these are the major isoforms, there are other FKBP and CyP that can inhibit CN, vary in tissue expression, and contribute to the effect of CsA and FK.

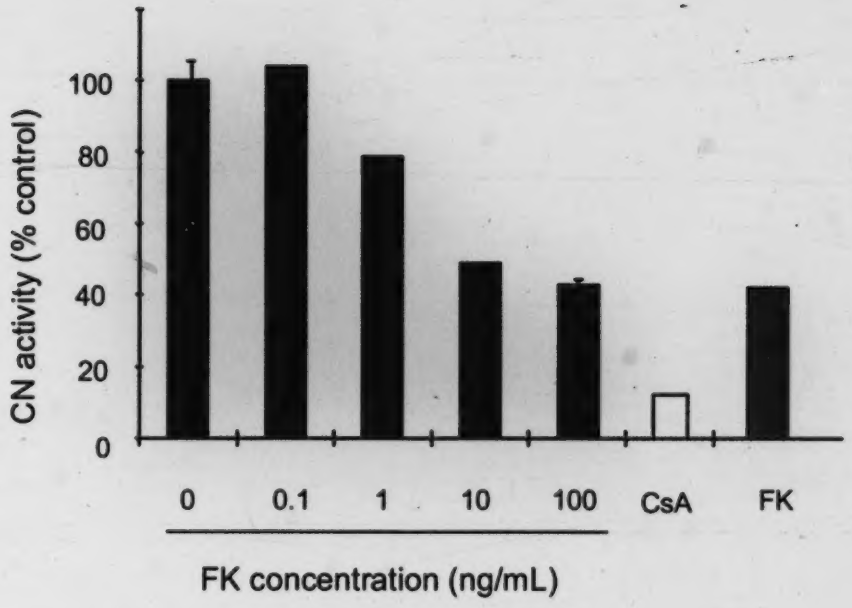


Figure 22. Lack of complete inhibition by FK and CsA is not substrate-specific. CN phosphatase activity against a serine-phosphorylated 31-mer peptide was measured in extracts from human PBL treated with FK in culture for 30 min at 37°C. Maximal inhibition of CN phosphatase activity was determined by adding CsA (1000 ng/mL) or FK (500 ng/mL) to the assay buffer. This experiment was performed twice.

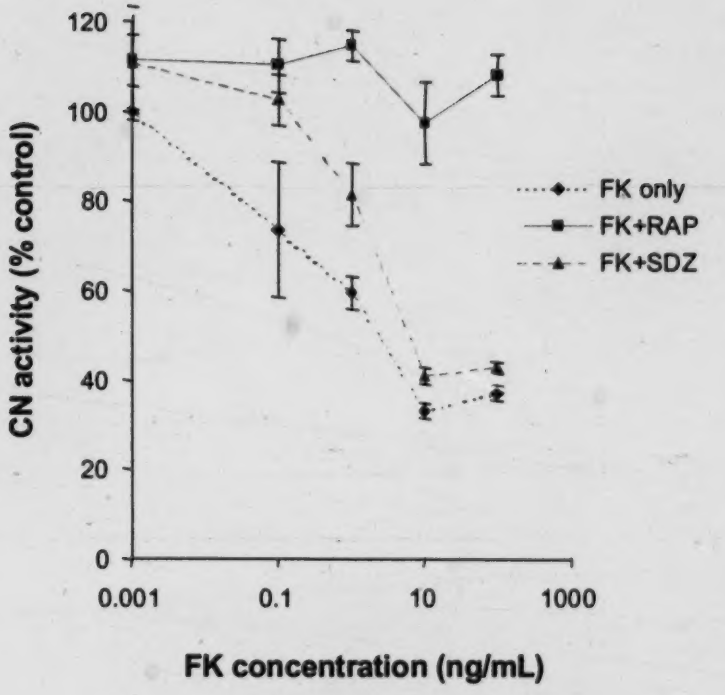


Figure 23. The specificity of the assay system as illustrated by rapamycin reversal of FK inhibition of CN phosphatase activity. Human PBL were treated with FK in culture for 30 min at 37°C followed by rapamycin or SDZ for another 30 minutes. Whole cell lysates were then assayed for CN activity, expressed as a percent of untreated PBL. This experiment was performed once.

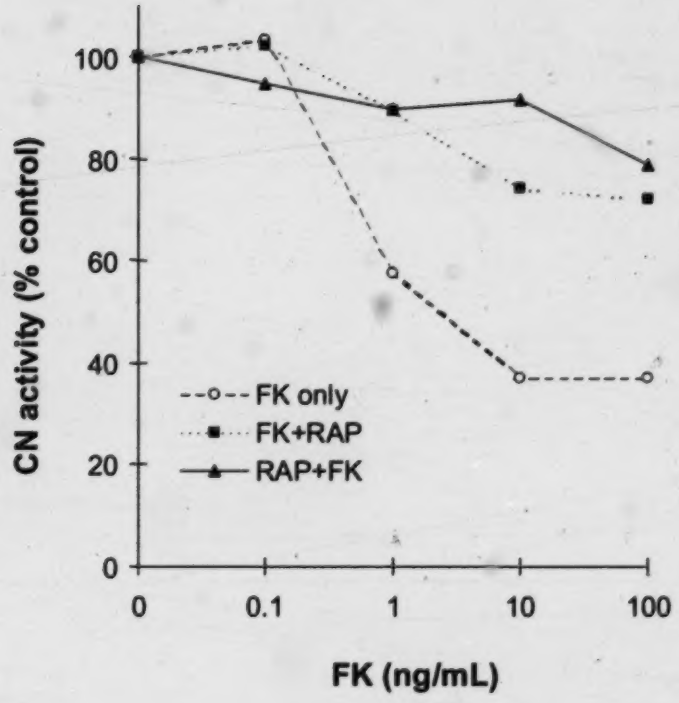


Figure 24. Rapamycin reversal of FK inhibition of CN phosphatase activity either before or after FK treatment. Human PBL were treated with FK only in culture for 60 min (FK only), FK for 30 min followed by rapamycin for 30 minutes (FK+RAP), or rapamycin for 30 min followed by FK for 30 min (RAP+FK). This experiment was performed once.

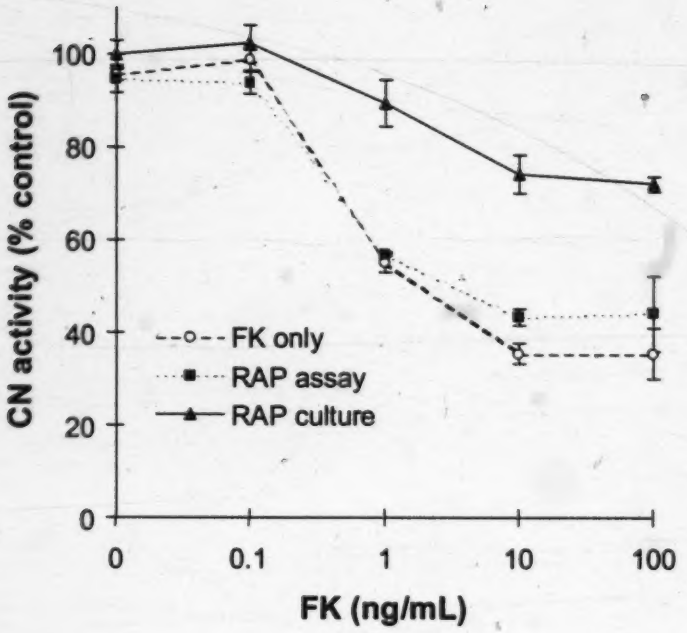


Figure 25. The reversal of FK inhibition of CN phosphatase activity by rapamycin. Human PBL were treated with FK in culture for 30 min and assayed in the absence (FK only) or presence (RAP assay) of added rapamycin in assay buffer, or PBL were treated with FK for 30 min followed by rapamycin for 30 minutes and assayed without added rapamycin (RAP culture). This experiment was performed once.

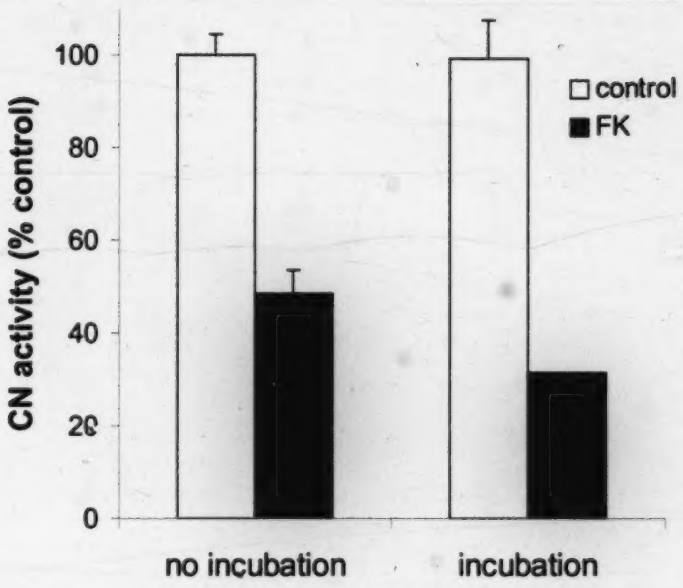


Figure 26. Pre-incubation with FK does not result in complete inhibition. Cell extracts from PBL were pre-incubated or not with FK for 5 min at 37°C. CN activity was calculated as a percent of the control, with no pre-incubation and no FK. This experiment was performed once.

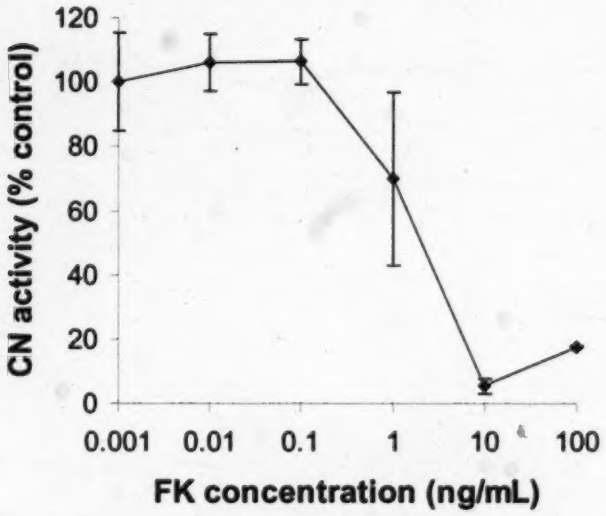


Figure 27. FK completely inhibits CN phosphatase activity in Jurkat T cells. Jurkat T cells were treated with FK for 30 min at 37°C and cell lysates were assayed for CN activity. CN activity was calculated as a percent of the control, with no FK. This experiment was performed twice.

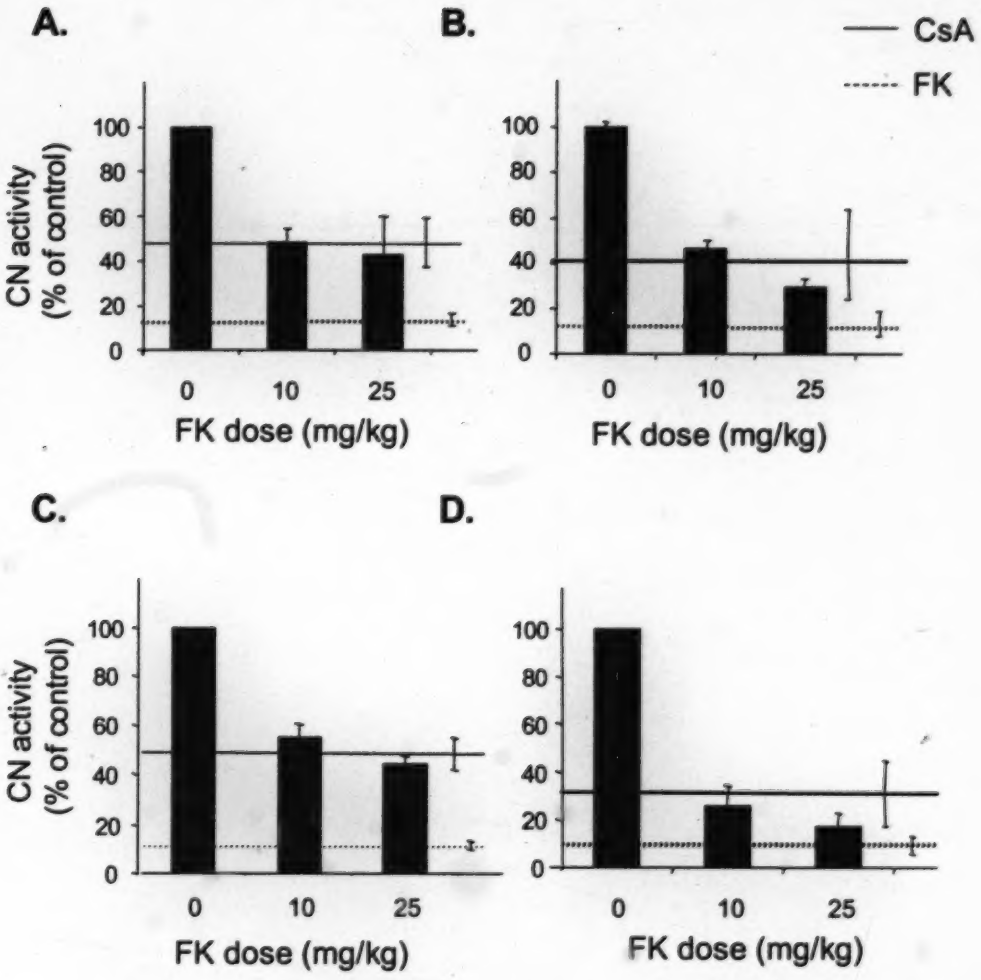


Figure 28. FK Inhibition of CN in mice *in vivo*. Mice were fed FK and tissues were harvested 1 h post does. Extracts from (a) whole blood, (b) spleen cell suspensions, (c) spleen homogenates, and (d) kidney homogenates were assayed for CN activity. CsA (1000 ng/mL) or FK (500 ng/mL) was added directly to the assay to determine the maximum inhibition by CsA and FK, respectively. CN activity was calculated as a percent of the control, with no FK. Each group consisted of four mice and samples were pooled for analysis.

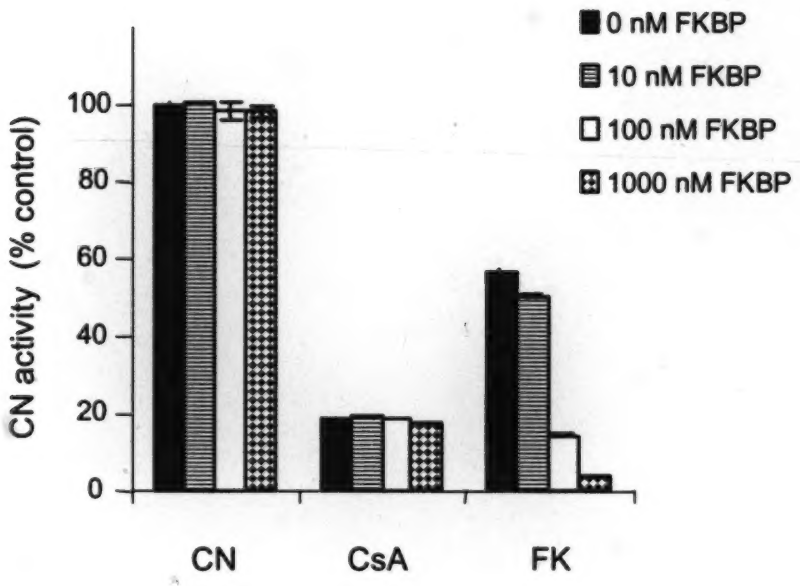


Figure 29. Exogenous FKBP12 specifically increases inhibition of CN by FK in a concentration-dependent manner. Human PBL were treated with CsA (1000 ng/mL) or FK (100 ng/mL) in culture and cell extracts were assayed for CN activity in the presence of increasing concentrations of exogenous FKBP12. CN activity was calculated as a percent of the control, with no CsA, FK, or FKBP12. This experiment was performed once.

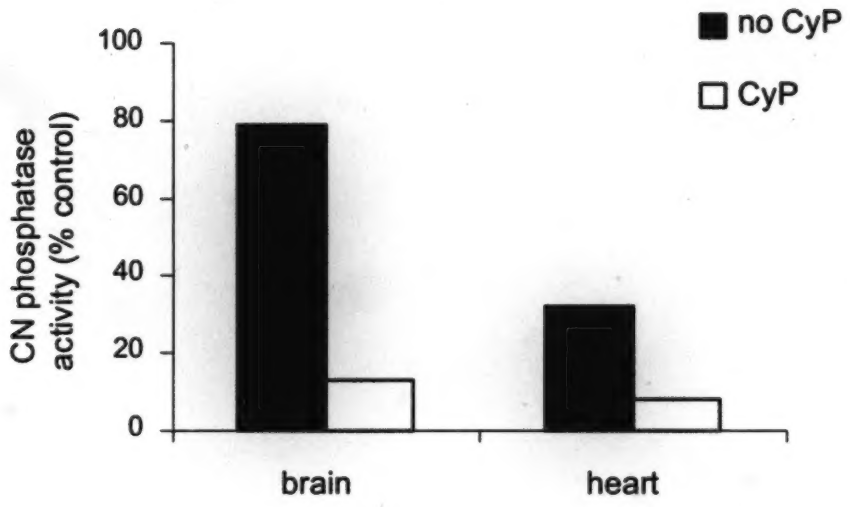


Figure 30. Inhibition of CN in mouse brain and heart homogenates is increased by exogenous CyPA. Extracts from mouse brain and heart homogenates were assayed for CN activity in the presence of CsA (1000 ng/mL) with or without added CyPA (1000 nM). CN activity was calculated as a percent of the control, with no CsA or CyPA. This experiment was performed once.

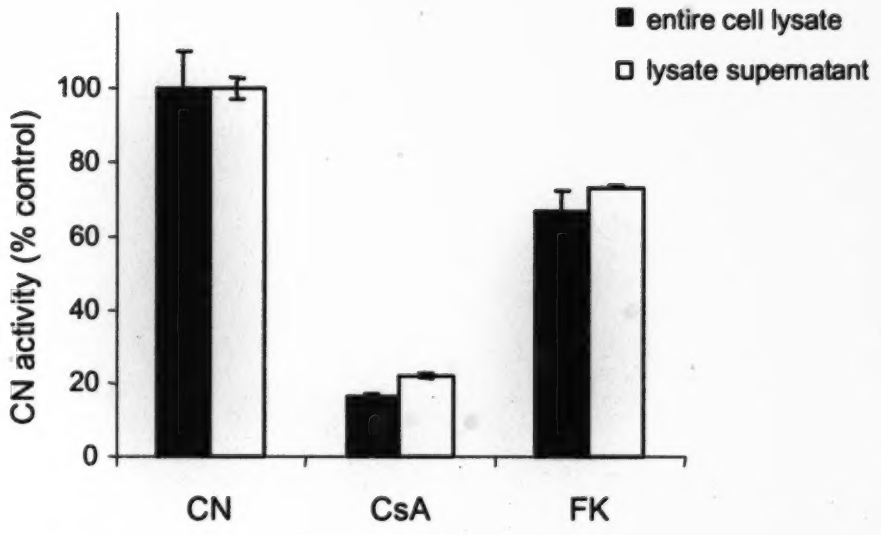


Figure 31. Comparison of inhibition of CN by CsA and FK in entire cell lysates and lysate supernatants. Mouse spleen cell suspensions were treated in culture with CsA (1000 ng/mL) or FK (100 ng/mL) and entire lysates or lysate supernatants were assayed for CN activity. CN activity was calculated as a percent of the control, with no CsA or FK. This experiment was performed once.

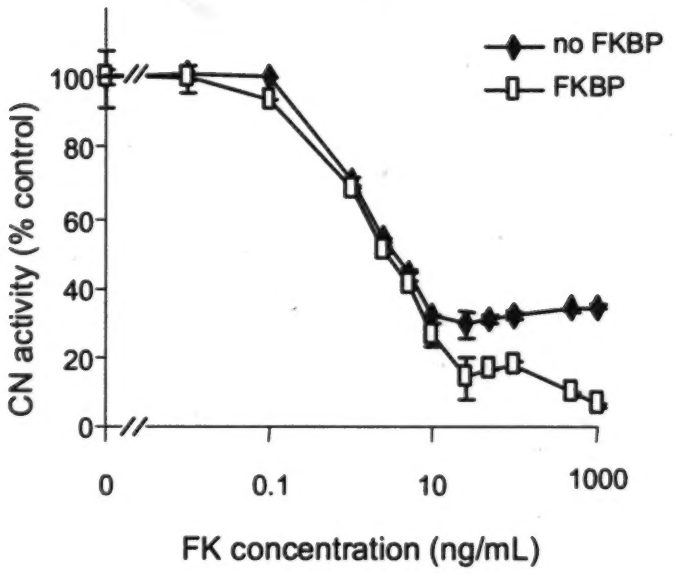


Figure 32. Comparison of inhibition of CN by FK in the presence or absence of exogenous FKBP12. Human PBL were treated in culture with increasing FK and cell extracts were assayed for CN activity in the presence or absence of exogenous FKBP12. CN activity was calculated as a percent of the control, with no FK or FKBP12. This experiment was performed once.

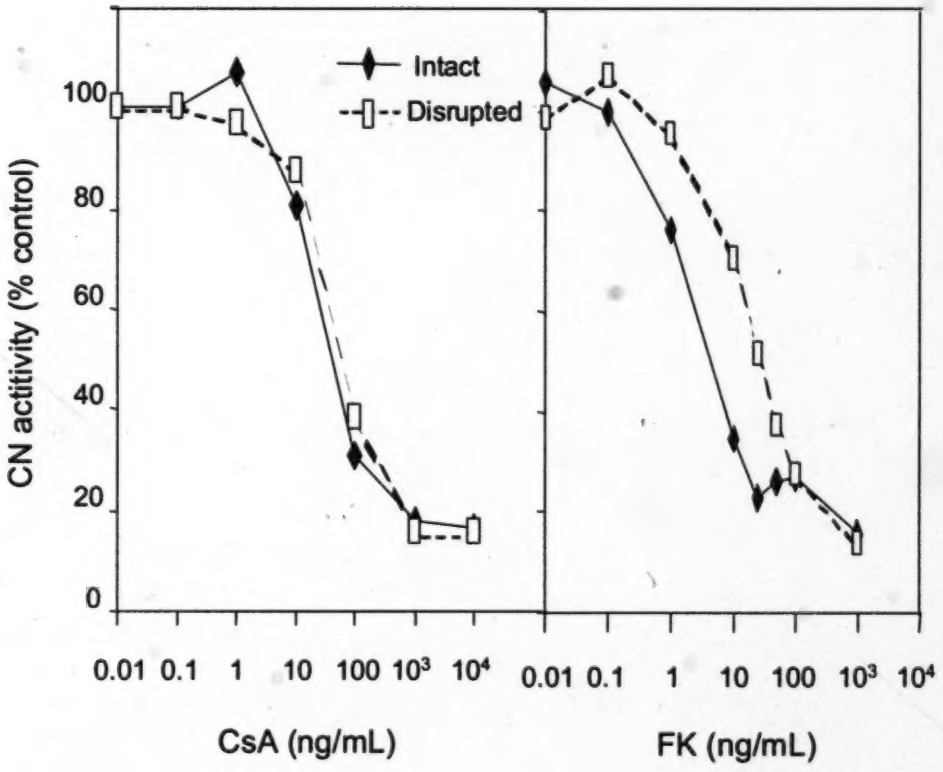


Figure 33. Inhibition of CN by CsA or FK in culture vs. in assay. PBL were treated in culture with CsA or FK and assayed in the presence of exogenous CyPA or FKBP12 (intact). Alternatively, extracts of PBL were assayed in the presence of CsA or FK in the presence of CyPA or FKBP12 (disrupted). CN activity was calculated as a percent of the control, with no CsA or FK. CyPA (1000 ng/mL) or FKBP12 (1000 ng/mL) is added to the assay buffer. This experiment is representative of two independent experiments.

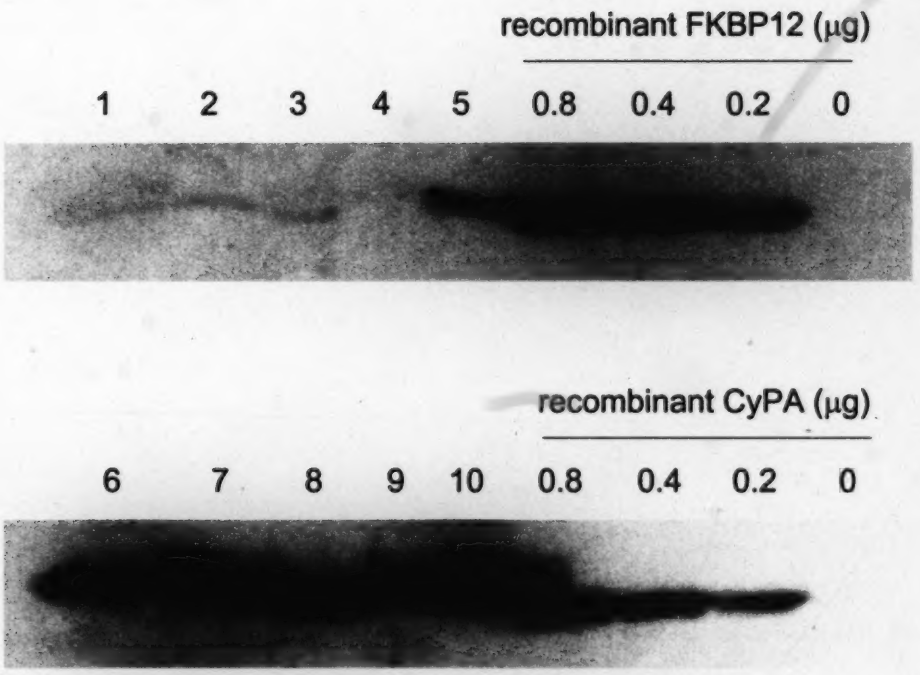


Figure 34. Expression of immunophilins in mouse tissues. Levels of FKBP12 (lanes 1-5) and CyPA (lanes 6-10) are determined by Western blot. Extracts from spleen (lanes 1 and 6), kidney (lanes 2 and 7), heart (lanes 3 and 8), liver (lanes 4 and 9), and brain (lanes 5 and 10) homogenized in RIPA buffer (1 g/ 3mL) were analyzed on a 15% SDS-PAGE gel. Recombinant CyPA and FKBP12 were included for approximation of levels in tissue homogenates. 10 μL of tissue extract (except for brain, 5 μL) was loaded in each lane. These blots are representative of two separate runs.

Table 2: Effect of exogenous FKBP12 on FK inhibition of CN in different mouse tissues

	control	FK			FK+FKBP		
	cpm [a]	cpm [a]	% control	% inhibition	cpm [a]	% control	% inhibition
spleen cell suspension [b]	27180 ± 766	19925 ± 108	73	27	1670 ± 52	6	94
brain homogenate [b]	45765 ± 373	34664 ± 91	76	24	2936 ± 91	6	94
whole blood [b]	10843 ± 690	5493 ± 174	51	49	353 ± 5	3	97

[a] Actual numbers from one experiment. Other experiments showed similar results.

[b] EGTA: spleen cell suspension, 4980 ± 81 cpm; brain homogenate, 1326 ± 37 cpm; mouse whole blood, 5163 ± 312 cpm

Table 3: The maximum inhibition of CN activity by FK in the presence or absence of endogenous FKBP12^

Tissue	FK max % inhibition	FK+FKBP max % inhibition
Spleen	42	95
Liver	60	95
Heart	49	94
Kidney	49	94
Brain	32	96

^These data represent two or three independent determinations.

Table 4: Comparison of *in vitro* IC50s of CsA and FK in mouse tissues^

Tissue	CsA *in vitro* IC50 (95% CI)	FK *in vitro* IC50 (95% CI)
Spleen	48 ng/mL (41-56)	44 ng/mL (30-64)
Liver	19 ng/mL (16-23)	41 ng/mL (36-47)
Heart	38 ng/mL (16-88)	51 ng/mL (39-68)
Kidney	26 ng/mL (21-32)	39 ng/mL (29-52)

^These data represents two or three independent determinations.

Chapter Five

Transforming Growth Factor (TGF) - β and Cyclosporine

A version of this chapter will be submitted for publication.

Kung, L., Loertscher, R., Melk, A., Sawitzki, B., Ramassar, V., and Halloran, P.F. The mechanism of cyclosporine is independent of transforming growth factor - β1.

A number of studies have attempted to examine the relationship between CsA and TGF-β1. While some studies conclude no correlation between CsA and TGF-β1 levels (267), other studies show stimulatory effects of CsA on TGF-β1 (268). The study of TGFβ presents some problems and its measurement is a topic of much debate (269). Many studies of TGF-β1 are limited by the analytical technique used or the possibility of artefacts resulting from sample preparation. Subsequently, I addressed more carefully the question of the effect of CsA on the levels of both latent and active TGF-β1 *in vitro* and *in vivo*.

The detection system, mode of TGF-β1 activation, use of serum in the culture media, and the dilution of the sample for the assay have all been shown to affect the analysis of TGF-β1 levels (270). Therefore, I used the R&D detection system (rated the highest), used serum free media where possible (various sources of serum are known to contain significant amounts of TGFβ-1), and limited dilution of samples (to reduce the amplification of any errors in measurement). In analyzing clinical samples, I assessed TGF-β1 in plasma rather than serum (since the clotting process results in the release of TGF-β1) and ensured that plasma samples were processed within 30 min of blood collection (mishandling of samples results in release of latent TGF-β1,

Figure 35). For mishandled samples where TGF-β1 levels are high, platelet factor (PF) – 4 may be used to correct for *ex vivo* TGF-β1 release (271;272).

I. Spleen cell suspensions *in vitro*

I first determined whether mouse spleen cells could be induced to secrete TGF-β1 in vitro. Spleen cells were harvested from BALB/c mice under sterile conditions and suspended at 1 million cells per mL in serum free culture media. Spleen cell suspensions were treated with 0, 10, or 1000 ng/mL of CsA for 30 min and stimulated for 48 hours with anti-CD3 antibody (5 μg/mL), PMA (10 ng/mL), ionomycin (0.5 μM), or PMA (10 ng/mL)/ionomycin (0.5 μM). I measured TGF-β1 in the cell supernatant by ELISA. The sandwich ELISA kit by R&D Systems detected only the active form of TGF-β1 in both mouse and human TGF-β1. Consequently, latent TGF-β in the supernatant was acid-activated and calculated as the difference between total TGF-β (acid-activated sample) and active TGF-β (not activated sample). Media alone was assessed for active and latent TGF-β1 and neither was detected. We did not observe induction or activation of TGF-β1 secretion under any condition of stimulation. Furthermore, CsA treatment did not result in secretion of active or latent TGF-β1 by

stimulated or unstimulated cells (Figure 36). As a control to ensure the cells in the system were viable, I also analyzed IFN-γ secretion by these cells. In contrast to TGF-β secretion, IFN-γ secretion was induced by stimulation and inhibited by CsA (Figure 37). Thus, mouse spleen cell suspensions could not be induced to secrete active or latent TGF-β1 following stimulation or CsA treatment *in vitro*.

II. Peripheral blood leukocytes *in vitro*

I then determined whether human PBL could be induced to secrete TGF-β1 *in vitro*. PBL were purified from buffy coats under sterile conditions and suspended in RPMI with 5% pooled human serum at 2 million per mL. RPMI+serum was used because I found that serum free media induced TGF-β secretion by resting PBL, resulting in even higher backgrounds (data not shown). Cells were stimulated with PMA (10 ng/mL)/ionomycin (0.5 μM) or anti-CD3 antibody (5 μg/mL) at 37°C. TGF-β1 protein in cell culture supernatants was assessed in cultures between 1 and 3 days. I found a time-dependent increase in levels of latent TGF-β1 following stimulation with PMA/ionomycin but not anti-CD3 antibody (Figure 38). TGF-β1 levels were below the level of detection without acid activation (data not shown).

To determine whether CsA alone or in conjunction with stimulation induced TGF-β1 secretion by human PBL, cell cultures were treated 0, 1, 10, 100, 1000, or 10000 ng/mL of CsA and stimulated or not with PMA/ionomycin or anti-CD3 antibody at 37°C. TGF-β1 protein in culture supernatants was measured at 48 hours by ELISA. The level of TGF-β1 protein was also not affected by CsA treatment (Figure 39). In contrast, CsA inhibited IFN-γ secretion in stimulated cells (Figure 40).

I next determined whether PMA or ionomycin alone induced TGF-β1 secretion by human PBL. Cell cultures were stimulated with PMA alone (10 ng/mL), ionomycin alone (0.5 μM), or PMA (10 ng/mL)/ionomycin (0.5 μM). TGF-β1 protein was measured at 48 hours (ELISA). I found that PMA alone but not ionomycin alone induced TGF-β1 secretion; furthermore, the induction by PMA alone was equivalent to that by PMA/ionomycin (Figure 41). As in the previous experiments, TGF-β1 levels were undetectable without acid activation. Thus, PMA alone but neither anti-CD3 nor ionomycin alone induced the secretion of latent TGF-β1 by human PBL. Furthermore, CsA did not induce nor increase latent TGF-β1 secretion.

III. The effect of CsA on TGF-β1 expression in mice *in vivo*

To examine whether CsA induced TGF-β1 protein expression *in vivo*, BALB/c mice were fed CsA (100 mg/kg/day) and kidneys were harvested at 1 h, 4 h and 24 h after the first dose and at d 7 after daily dosing. Sham mice were fed olive oil daily and harvested on d 7. The expression of TGFβ1 mRNA in the kidney was assessed using the RNAse protection assay. Briefly, RNA was extracted from individual kidneys and hybridized overnight to the ^{32}P-labeled anti-sense RNA probe that was synthesized from the supplied DNA template set (mCK-3 from PharMingen). Single-stranded RNA and free probe were digested by RNase A and T1. Protected RNA was phenolized, precipitated, and resolved on a 5% denaturing polyacrylamide gel. Kodak X-Omat AR film was exposed to the gel for 15 minutes with two intensifying screens at -70°C and the quantity of protected RNAs was determined using a phosphor imager. TGF-β1 transcripts were identified by the length of the respective fragments. For quantitation, TGF-β1 values were expressed as a percentage of the values of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each gel lane. Two mice were averaged per group. I found no change in the expression of TGF-β1 mRNA in mice fed a high dose of CsA daily for 7 days (Figure 42). I confirmed these results using real time PCR (Taqman) (Figure 43). Three (4 h, d 1, d 7) or

eight (sham, 1 h) mice were averaged per group and TGF-β1 mRNA expression was normalized to β-actin and expressed relative to the sham (vehicle-treated) mice.

Although I did not find any induction of TGF-β1 mRNA by CsA up to d 7, it was possible that CsA treatment resulted in the activation of latent TGF-β1. To examine this possibility, I stained for active TGF-β1, latent TGF-β1, and collagen IV in frozen kidney sections. The TGF-β1 antibodies that I chose to use have been previously shown to distinguish between the active and latent forms of TGF-β1 using immunofluorescence in frozen sections of mouse mammary gland (247;273;274).

Cisplatin is a chemotherapeutic agent that also induces kidney injury (275) and thus could serve as a positive control for the staining system. Frozen sections of kidneys from mice injured with cisplatin (20 mg/kg, d 7; contributed by Joan Urmson) were stained with either normal isotype control or TGF-β1 specific antibodies. Staining conditions (incubation time, blocking, etc.) were systematically adjusted until there was little or no staining with the normal isotype control compared with the specific antibodies. As shown in Figure 44, the normal goat IgG gave some light nonspecific staining which increased slightly with cisplatin treatment. In comparison, the specific antibody for latent TGF-β1 gave moderate tubular staining that

increased intensely following cisplatin treatment (Figure 45). This specific tubular staining is particularly evident when the section is examined at a higher magnification (Figure 46). In contrast to the normal goat IgG, normal chicken IgY gave very little if any background staining in both the sham and cisplatin-treated sections (Figure 47). Tubules in the cisplatin-treated kidneys were slightly more stained by the antibody specific for active TGF-β than the sham-treated kidneys (Figures 48 and 49).

I then examined the effect of CsA on the *in vivo* protein expression of TGF-β1. Mice were fed 100 mg/kg of CsA daily for 7 days. Kidneys were harvested post-dose at 1 h, 4 h, d 1, and d 7. Frozen kidney sections were stained with antibodies specific for latent TGF-β1 (Figures 50 and 51) or active TGF-β1 (Figures 52 and 53). Only sham, 4 h, d 1, and d 7 sections are shown. Unlike with cisplatin treatment, there was no increase in tubular staining for both latent and active TGF-β1 over the 7 days of CsA treatment. Thus, CsA does not increase the protein expression of latent or active TGF-β1 over a period of 7 days.

IV. The effect of immunosuppressive regimens on TGF-β1 levels in humans.

I also conducted a human population study examining TGF-β levels in relationship to the actions of immunosuppressive drugs and to determine whether there are higher TGF-β levels in patients on CN-inhibitor therapy compared to patients on other immunosuppressive therapies. I collected whole blood from patients and normal volunteers and measured active (unactivated sample) and total (acid-activated sample) TGF-β1 levels in the plasma. The assessment of TGF-β1 in serum is not recommended because the majority of TGF-β1 detected in serum is released from platelets during the clotting process (270). All blood was collected using EDTA as an anticoagulant and processed within 30 minutes of collection. These are measures that have been shown to reduce the *ex vivo* induction of TGF-β1. The level of platelet factor 4 (PF-4) was also measured in the same samples to control for changes in TGF-β1 due to sample mishandling and *ex vivo* platelet degranulation. This method of correction has been previously validated (271).

The average level and range of total plasma TGF-β1 in the normal group (7.3 ± 3.5 ng/mL) was comparable to those previously published (271;276). Furthermore, TGF-β1 levels in transplant patients did not differ from the normal population (Figure 54, Table 5).

The average in patients on CN-inhibitor therapy was 7.9 ± 5.3 ng/mL (1.6-23.4 ng/mL) while those on other immunosuppressive therapies had levels averaging 9.4 ± 9.3 ng/mL (2.7-43.5 ng/mL). Analysis of active TGF-β1 in plasma also showed no significant difference between transplant patients on immunosuppression and the normal population (Figure 55, Table 5). Levels in normals, CN-inhibitor therapy patients, and other immunosuppressive therapy patients were 44 ± 13 pg/mL, 37 ± 15 pg/mL, and 49 ± 39 pg/mL, respectively (Table 5). Measurement of PF-4 levels in the same samples showed no significant variation (Figure 56). These data is in agreement with other recent studies (267;276;277).

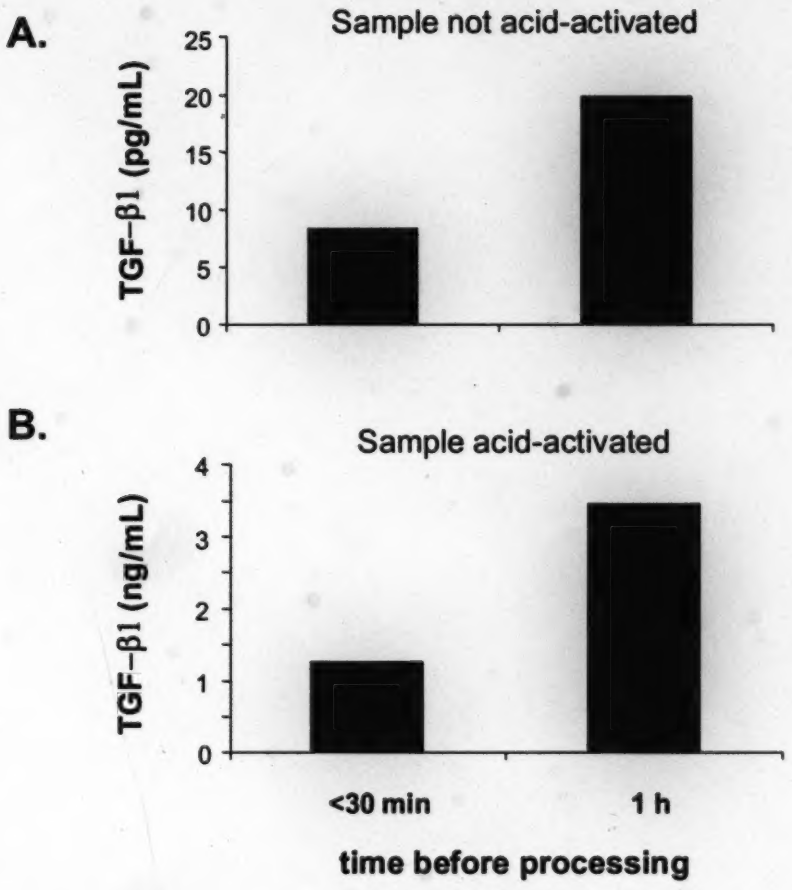


Figure 35. Effect of time before between collection of whole blood and processing of plasma on TGF-β1 in human whole blood. Two tubes of whole blood were collected by venous puncture and the processing of plasma from whole blood was commenced either <30 min or 1 h after collection. Plasma samples were not acid-activated (panel A) or acid-activated (panel B) and assayed for TGF-β1 levels. This experiment was performed once.

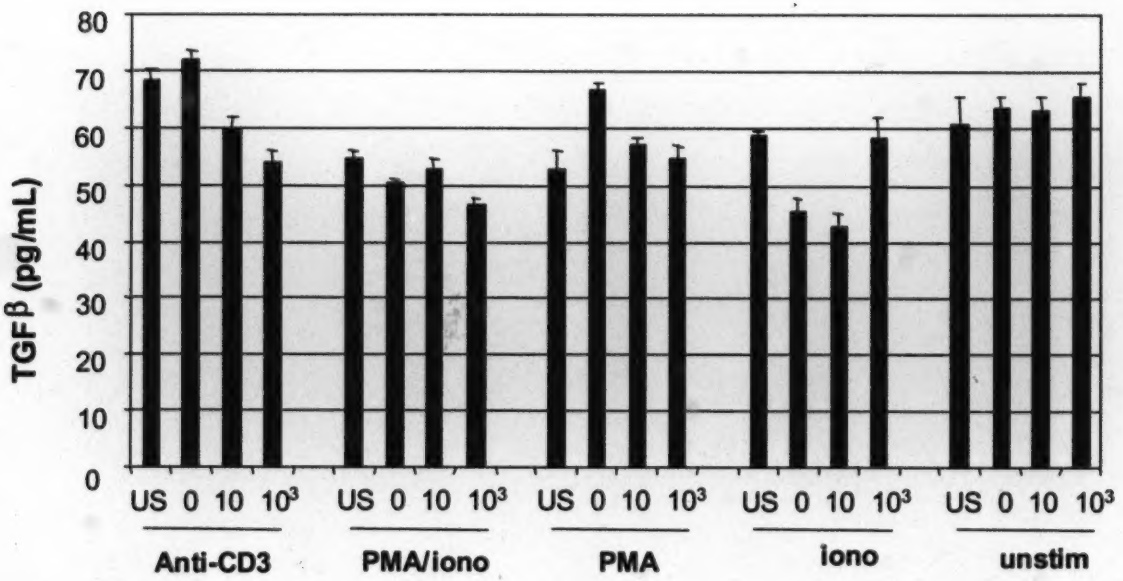


Figure 36 . CsA treatment and stimulation of spleen cell suspensions. Spleen cells were treated with 0, 10, or 1000 ng/mL CsA for 30 min and then stimulated or not with anti-CD3 (5 μg/mL), PMA (10 ng/mL)/ionomycin (0.5 μM), PMA (10 ng/mL), or ionomycin (0.5μM) for 48 hours. Cell culture supernatants were acid-activated and TGF- β1 was measured by ELISA. Each bar represents the mean of two separate determinations.

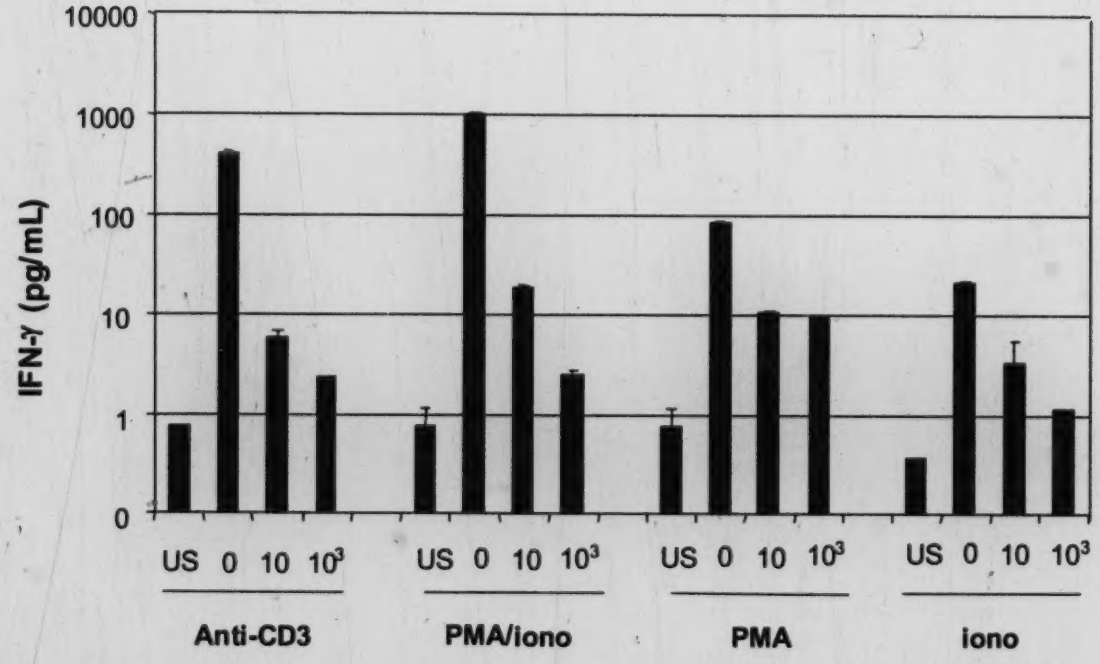


Figure 37. The effect of CsA and stimulation on IFN-γ secretion by spleen cell suspensions. Spleen cells were treated with 0, 10, or 1000 ng/mL CsA for 30 min and then stimulated or not with anti-CD3 (5 μg/mL), PMA (10 ng/mL)/ionomycin(0.5 μM), PMA (10 ng/mL), or ionomycin (0.5 μM) for 48 hours. IFN-γ in cell culture supernatants was measured by ELISA. Each bar represents the mean of two separate determinations.

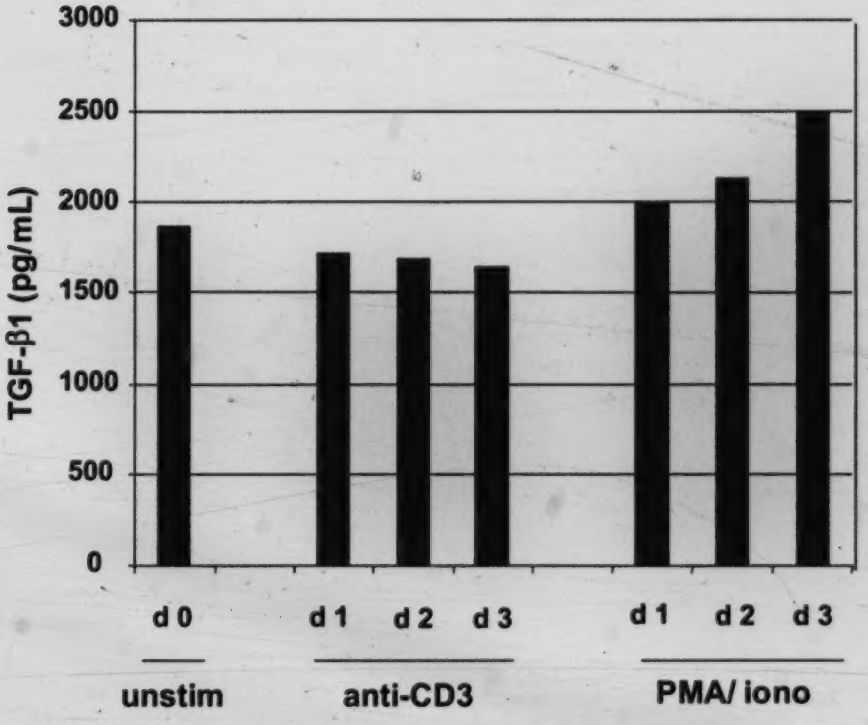


Figure 38. Effect of stimulation on latent TGF-β1 secretion by PBL. PBL were cultured in RPMI + 5% FBS and stimulated or not with anti-CD3 (5 μg/mL) or PMA (10 ng/mL)/ionomycin (0.5 μM). Cell culture supernatants acid-activated, diluted 1/5, and analyzed for TGF-β1 protein by ELISA at d 0, d 1 ,d 2, and d 3. Levels shown have been corrected for dilutions. This experiment was done once.

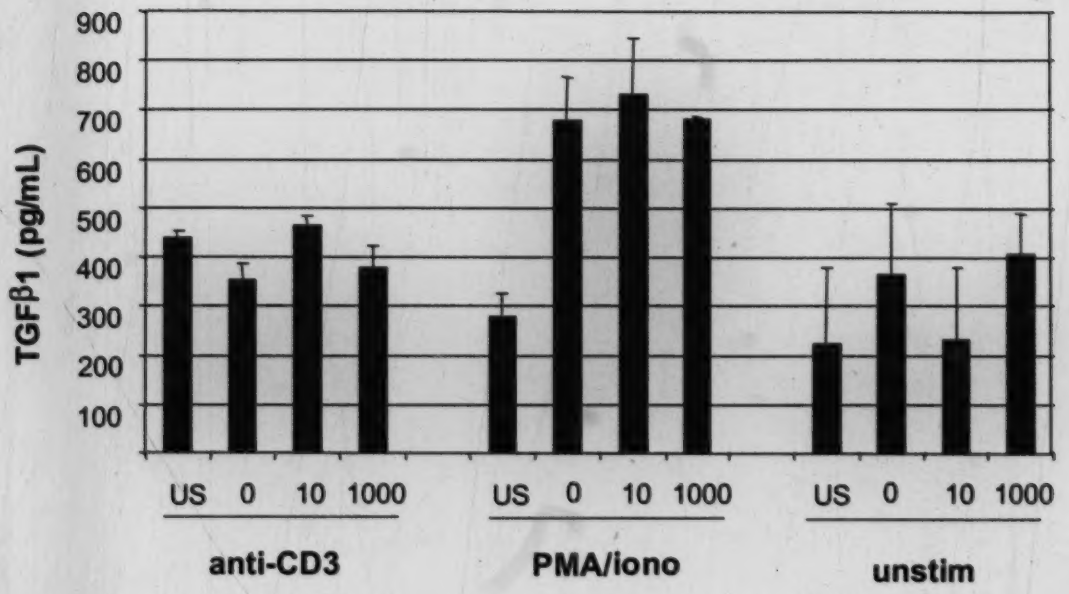


Figure 39. The effect of CsA and stimulation on the secretion of TGF-β1 by PBL. PBL were treated with 0, 10, or 1000 ng/mL CsA and stimulated or not with anti-CD5 (5 μg/mL) or PMA (10 ng/mL)/ ionomycin (0.5 μM). Cell culture supernatants were acid-activated and analyzed for TGF-β 1 protein at 48 h. The background level of TGF-β1 from the media alone was subtracted from all groups.

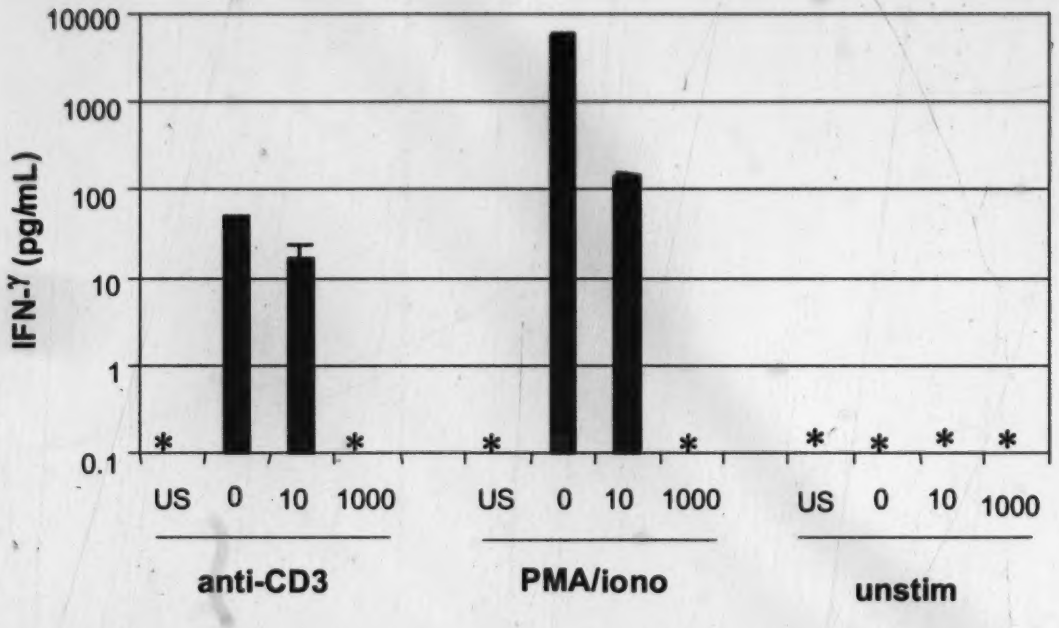


Figure 40. The effect of CsA and stimulation on the IFN-γ secretion by PBL. PBL were treated with 0, 10, 1000 ng/mL CsA and stimulated or not with anti-CD3 (5 μg/mL) or PMA 10 ng/mL/ionomycin (0.5 μM). Cell culture supernatants were analyzed for IFN-γ protein at 48 h.

* denotes undetectable levels.

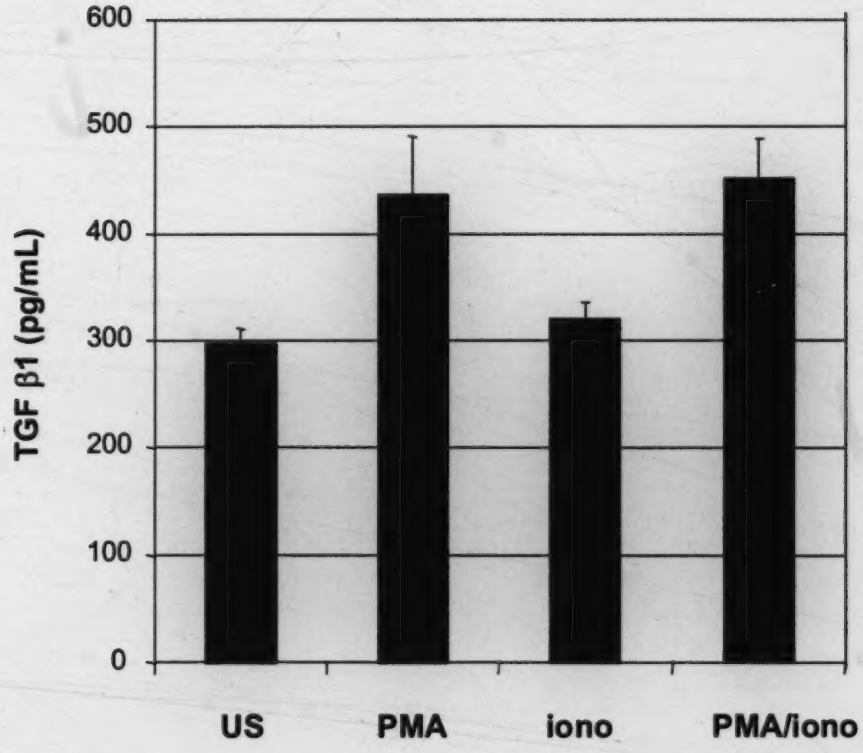
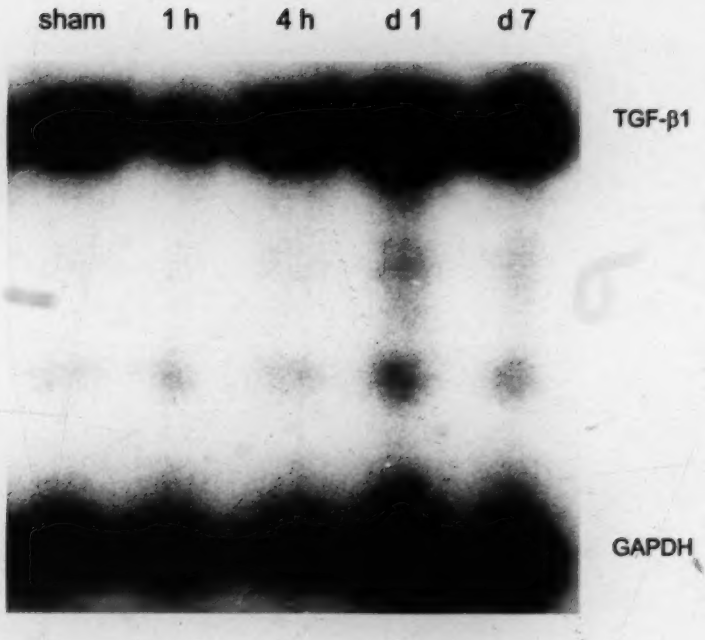


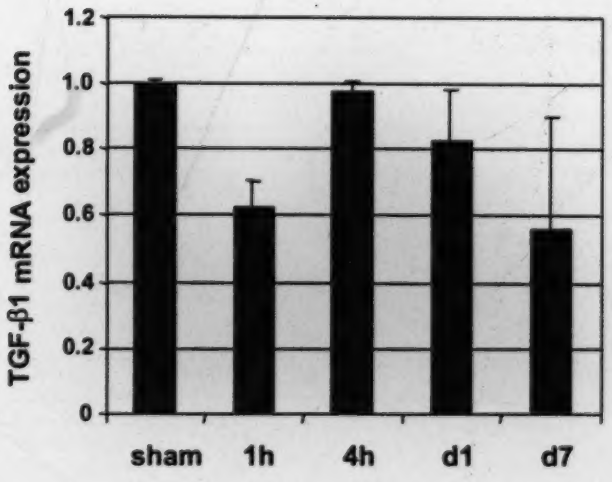
Figure 41. Effect of PMA and ionomycin on TGF-β1 secretion by PBL. PBL were stimulated or not with anti-CD3 (5 μg/mL), PMA alone (10 ng/mL), ionomycin alone (0.5 μM), or PMA (10 ng/mL)/ionomycin (0.5 μM). TGF-β1 levels were analyzed in cell culture supernatants at 48 h by ELISA. The average of duplicate determinations is shown for each group. Background levels of TGF-β1 from media alone have been subtracted from the values shown. This experiment was performed once.

Figure 42. TGF-β1 mRNA expression *in vivo* following CsA treatment. BALB/c mice were administered 100 mg/kg CsA daily for 7 d. Kidneys were harvested at 1 h, 4 h, and d 1 after the first dose and at d 7 after daily dosing. Sham animals were treated daily with olive oil and harvested on d 7. Total RNA was extracted and RNase protection assays were performed as described. The gel (panel A) is representative of two mice. TGF-β1 values were expressed as a percentage of GAPDH values and averaged for each time point (panel B). The Y-axes depict arbitrary Phosphor Imaging counts.

A.


sham
1 h
4 h
d 1
d 7
TGF-β1
GAPDH

B.


TGF-β1 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
sham
1h
4h
d1
d7

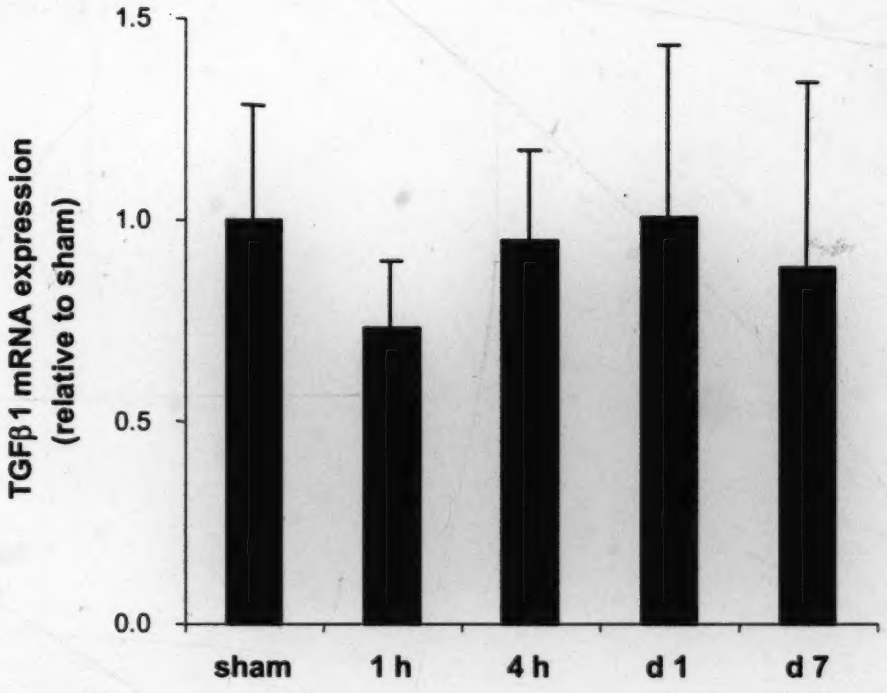
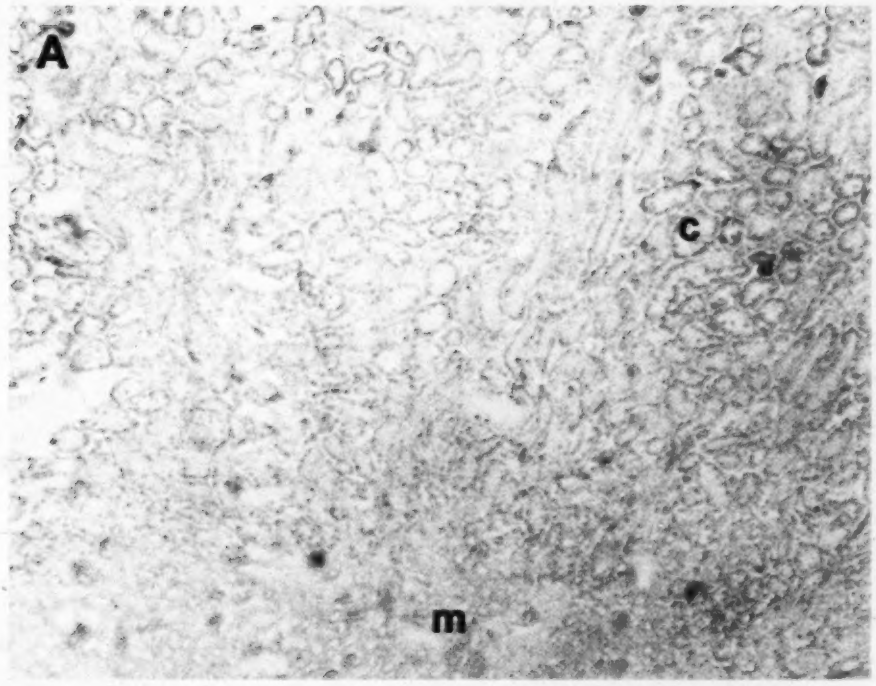


Figure 43. TGF-β1 mRNA expression *in vivo* following CsA treatment: analysis by real time PCR. BALB/c mice were administered 100 mg/kg CsA daily for 7 d. Kidneys were harvested at 0 h, 1 h, 4 h, d 1, and d 7. Total RNA was extracted and real time PCR (Taqman) was performed as described. TGF-β1 values were corrected using β-actin controls and groups were expressed relative to the sham. There were three or eight mice per group and mice were analyzed individually.

Figure 44. Photomicrographs of negative control antibody staining matched for latent TGF-β1 staining in mouse kidney. Frozen sections from sham treated (panel A) and cisplatin treated (panel B) were stained with normal goat IgG. The medulla (m) and cortex (c) are indicated. Both panels are the same magnification (original 100X). Tissue sections from an experiment performed by Joan Urmson. These slides are representative of two separate staining experiments.


A
c
m

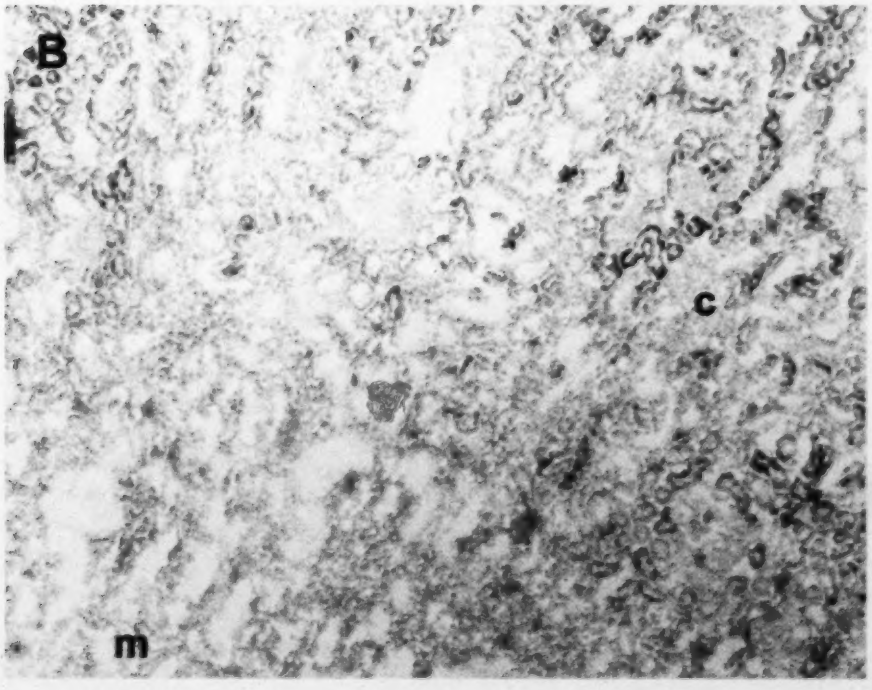

B
c
m

Figure 45. Photomicrographs of latent TGF-β1 staining in mouse kidney. Frozen sections from sham treated (panel A) and cisplatin treated (panel B) were stained with goat anti-LAP (latent TGF-β1) affinity purified polyclonal antibody. The medulla (m) and cortex (c) are indicated. Both panels are the same magnification (original 100X). Tissue sections from an experiment performed by Joan Urmson. These slides are representative of two separate staining experiments.

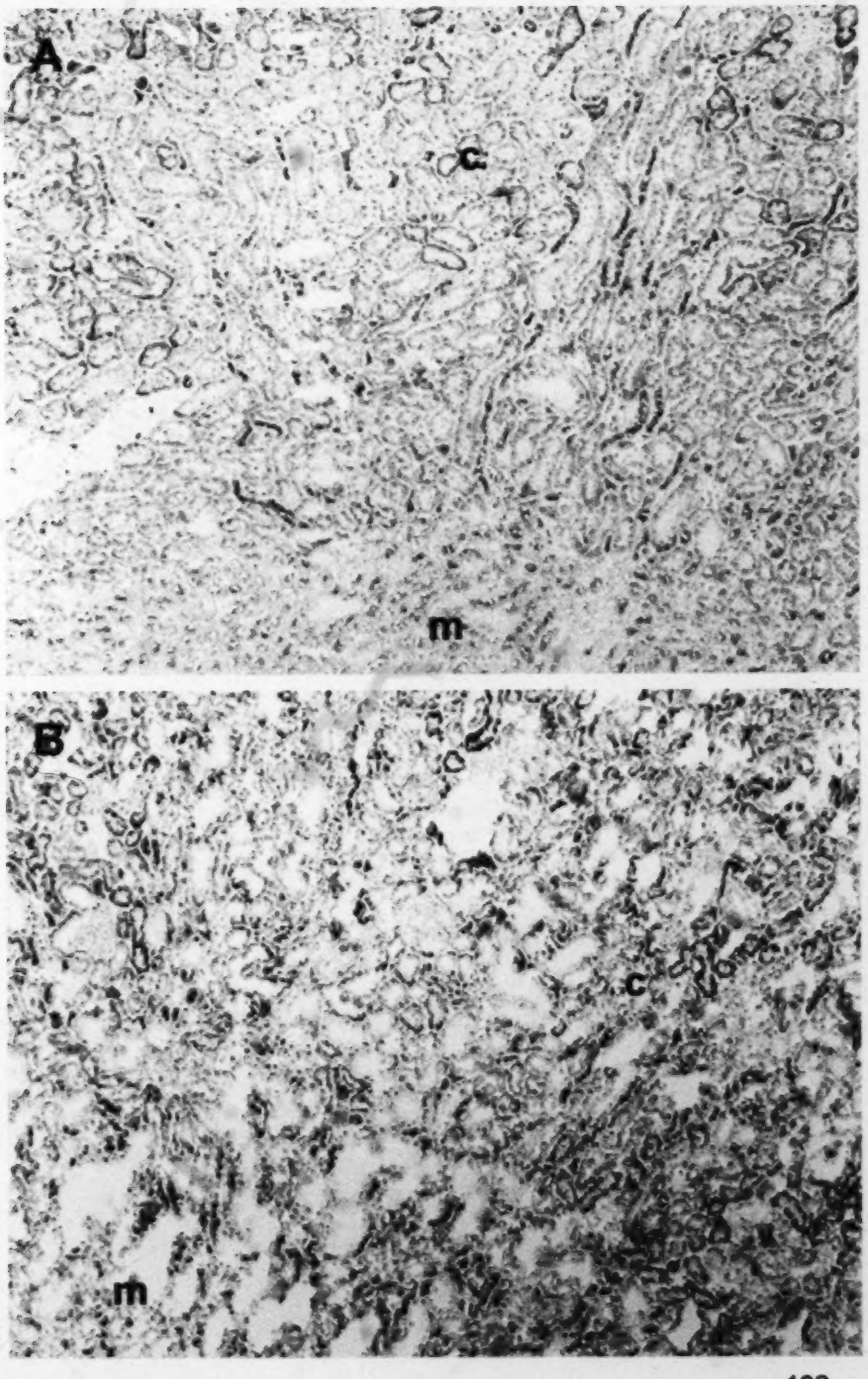

A
c
m
B
c
m

Figure 46. Magnification of latent TGF-β1 staining in mouse kidney following cisplatin-induced injury. Frozen sections from a cisplatin treated mouse were stained with normal goat IgG (panel A) or goat anti-LAP affinity purified polyclonal antibody (panel B). Both panels are the same magnification (original 400X). Tissue sections from an experiment performed by Joan Urmson. These slides are representative of two separate staining experiments.

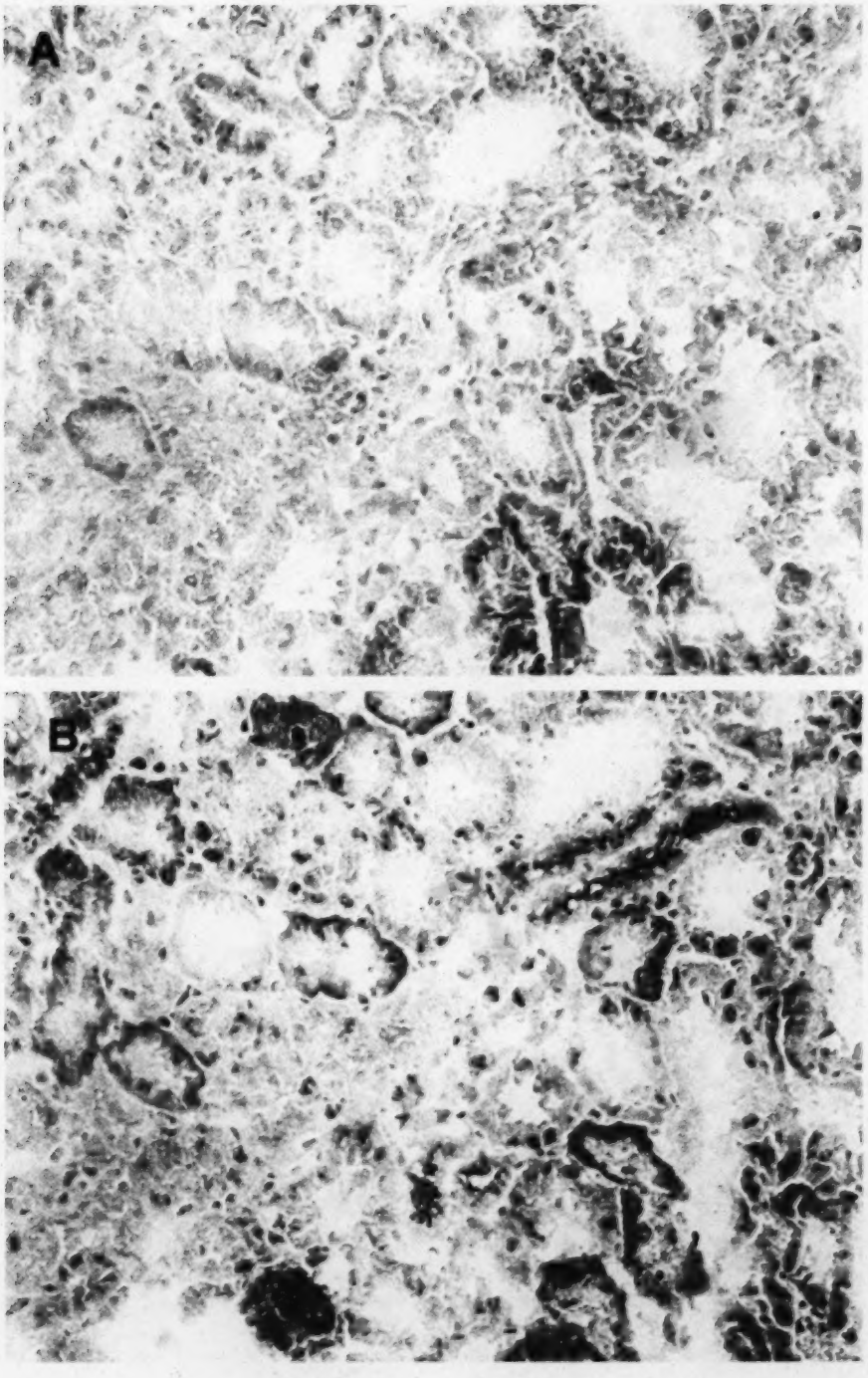

A
B

Figure 47. Photomicrographs of negative control antibody staining matched for active TGF-β1 staining in mouse kidney. Frozen sections from sham treated (panel A) and cisplatin treated (panel B) were stained with normal chicken IgY. The medulla (m) and cortex (c) are indicated. Both panels are the same magnification (original 100X). Tissue sections from an experiment performed by Joan Urmson. These slides are representative of two separate staining experiments.

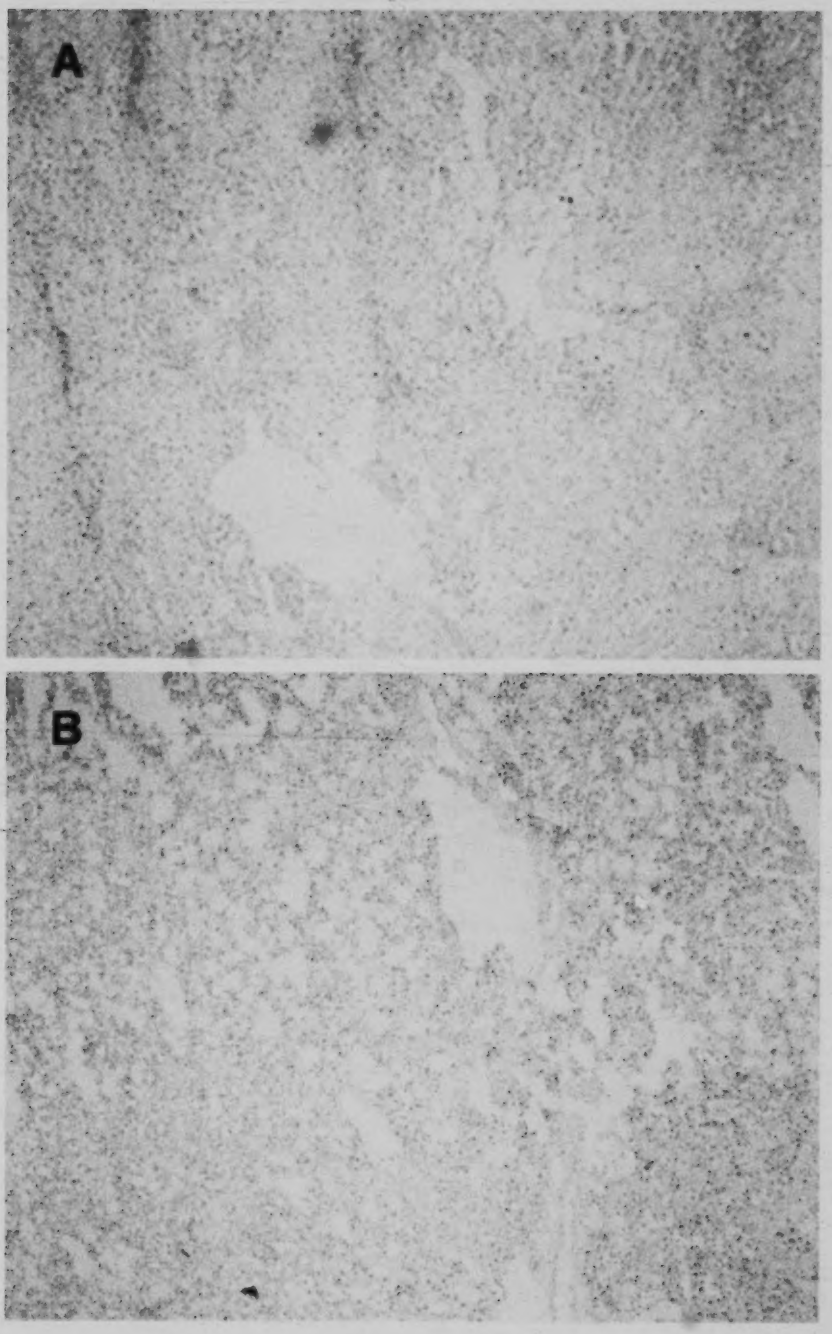

A
B

Figure 48. Photomicrographs of active TGF-β1 staining in mouse kidney. Frozen sections from sham treated (panel A) and cisplatin treated (panel B) were stained with anti-TGF-β1 affinity purified chicken polyclonal antibody. Both panels are the same magnification (original 100X). Tissue sections from an experiment performed by Joan Urmson. These slides are representative of two separate staining experiments.

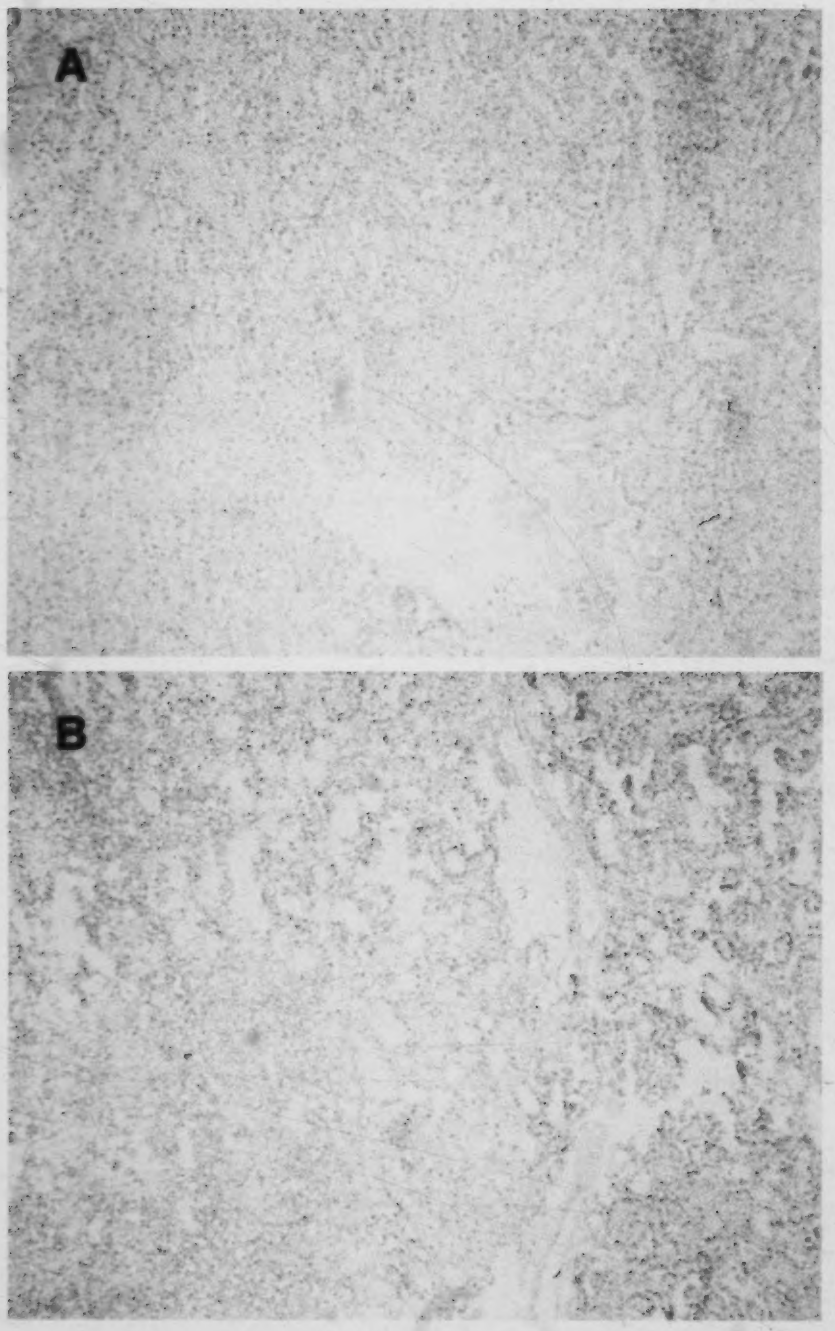

A
B

Figure 49. Magnification of active TGF-β1 staining in mouse kidney following cisplatin treatment. Frozen sections from a cisplatin treated mouse were stained with normal chicken IgY (panel A) or anti-TGF-β1 affinity purified chicken polyclonal antibody (panel B). Both panels are the same magnification (original 400X). Tissue sections from an experiment performed by Joan Urmson. These slides are representative of two separate staining experiments.

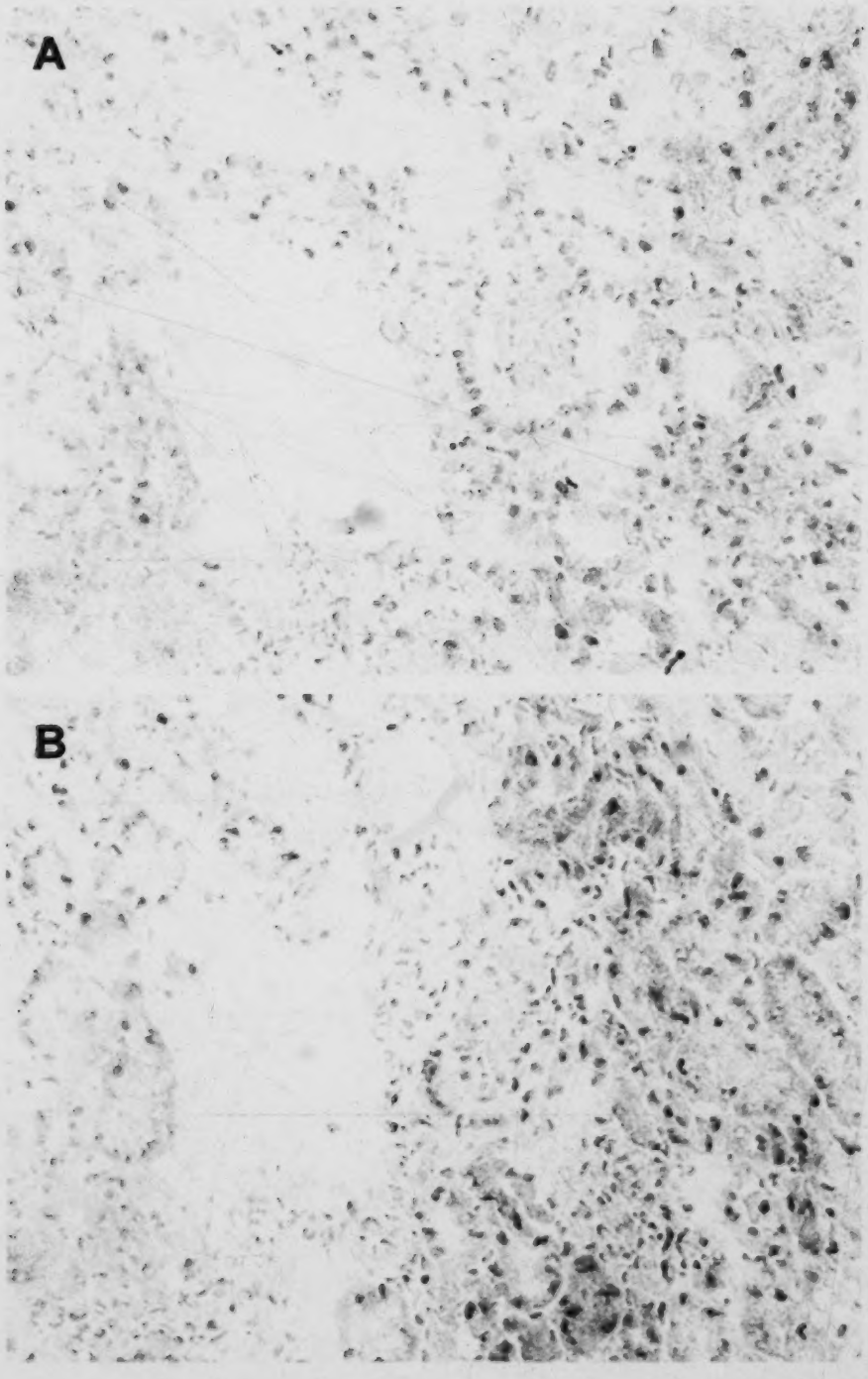

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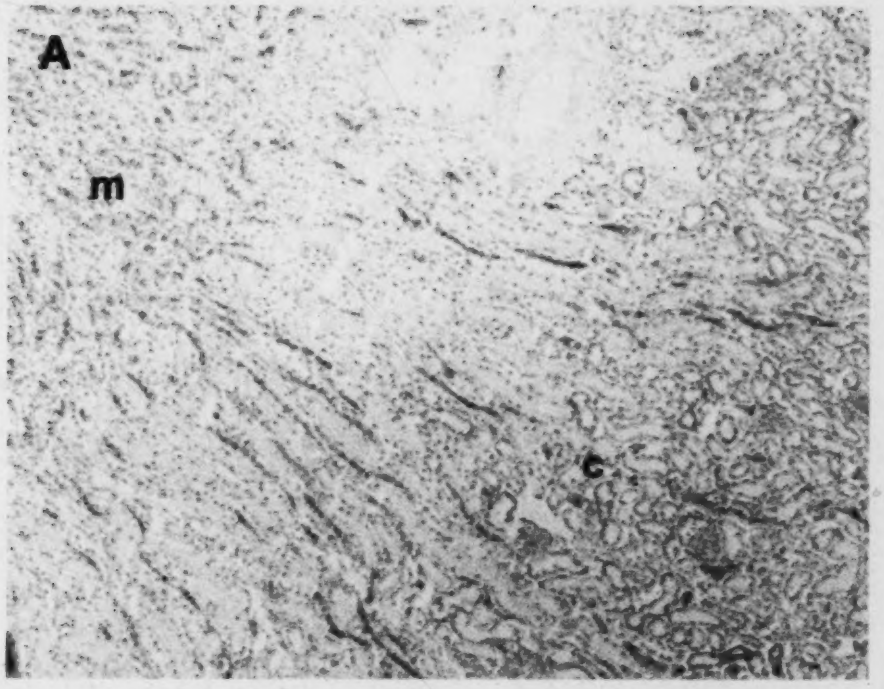
Figure 50. Photomicrographs of latent TGF-β1 staining in kidneys from mice administered CsA (sham and 1 h post-CsA). BALB/c mice were administered 100 mg/kg CsA daily for 7 d. Frozen sections from sham treated (panel A) and CsA treated (panel B) were stained with anti-LAP (latent TGF-β1) affinity purified goat polyclonal antibody. Sham kidney was harvested at d 7 while the CsA treated kidney was harvested at 1 h. The medulla (m) and cortex (c) are indicated. Both panels are the same magnification (original 100X). These slides are representative of two separate staining experiments.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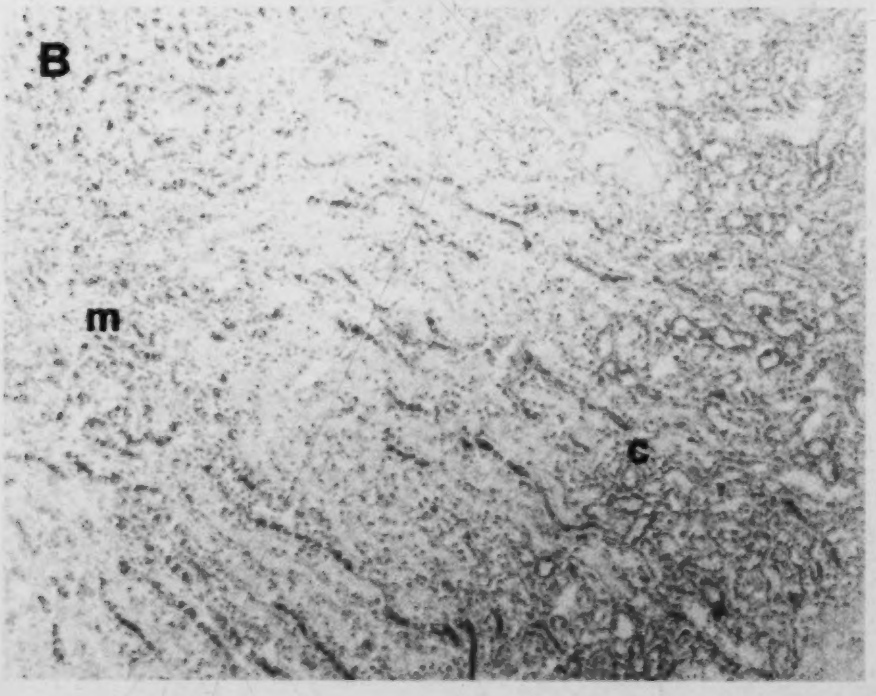
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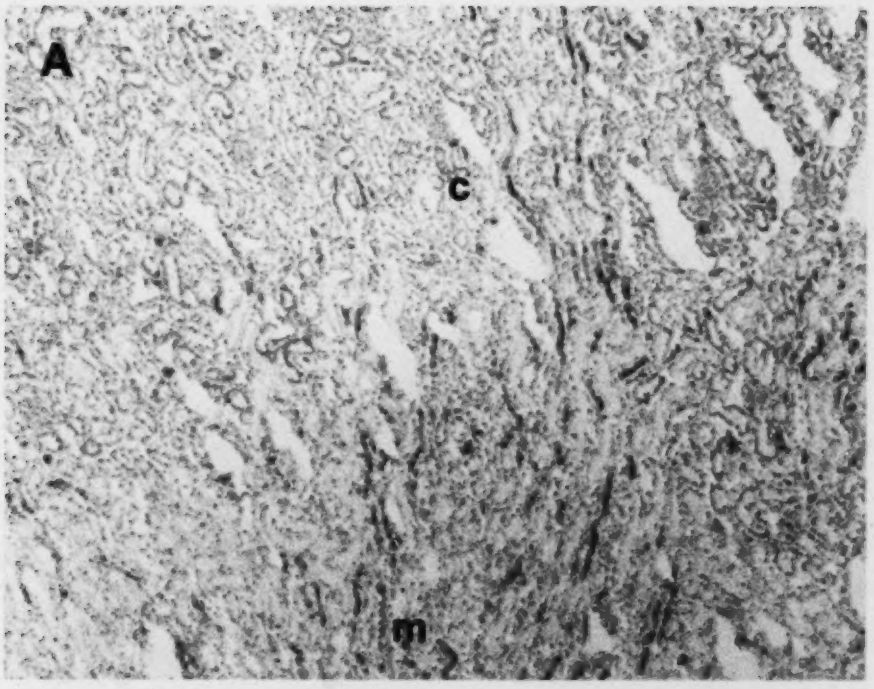
Figure 51. Photomicrographs of latent TGF-β1 staining in kidneys from mice administered CsA (d 1 and d 7 post-CsA). BALB/c mice were administered 100 mg/kg CsA daily for 7 d. Frozen sections from CsA treated mice were stained with anti-LAP (latent TGF-β1) affinity purified goat polyclonal antibody. Kidneys were harvested at d 1 (panel A) or d 7 (panel B). The medulla (m) and cortex (c) are indicated. Both panels are the same magnification (original 100X). These slides are representative of sections from three mice.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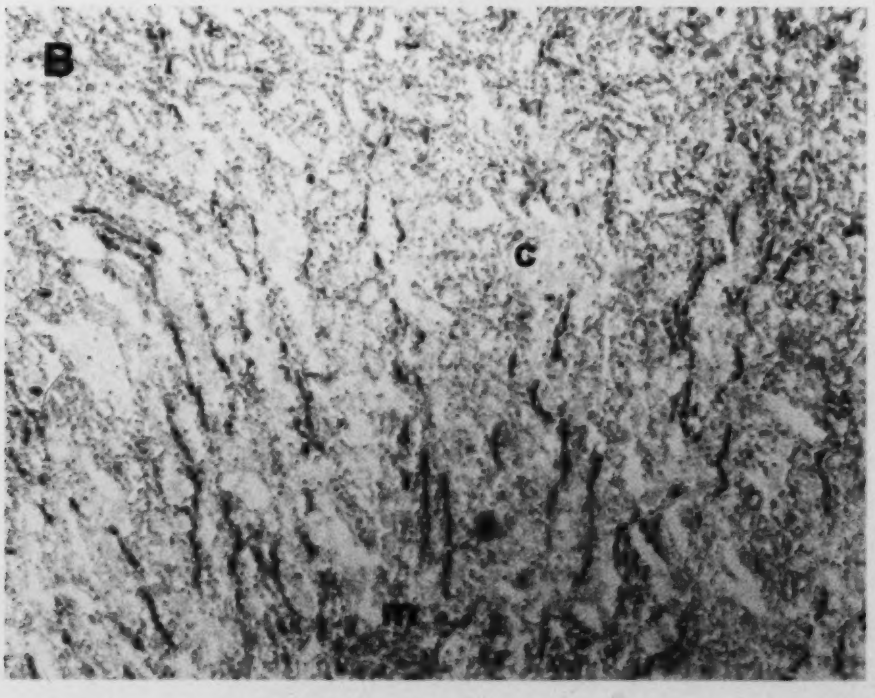
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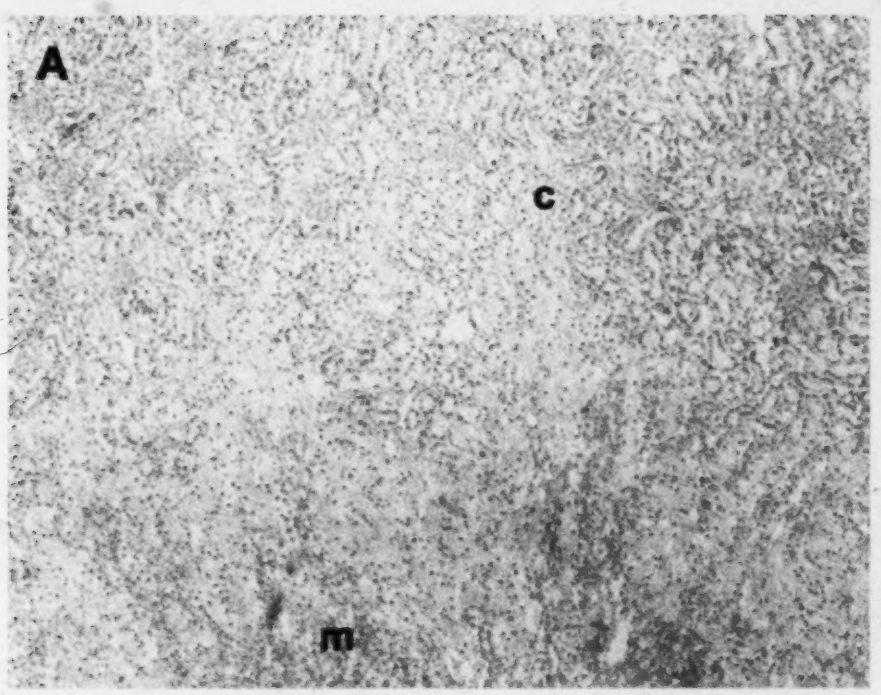
Figure 52. Photomicrographs of active TGF-β1 staining in kidneys from mice administered CsA (sham and 1 h post-CsA). BALB/c mice were administered 100 mg/kg CsA daily for 7 d. Frozen sections from sham treated (panel A) and CsA treated (panel B) were stained with anti-TGF-β1 affinity purified chicken polyclonal antibody. Sham kidney was harvested at d 7 while the CsA treated kidney was harvested at 1 h. The medulla (m) and cortex (c) are indicated. Both panels are the same magnification (original 100X). These slides are representative of sections from three mice.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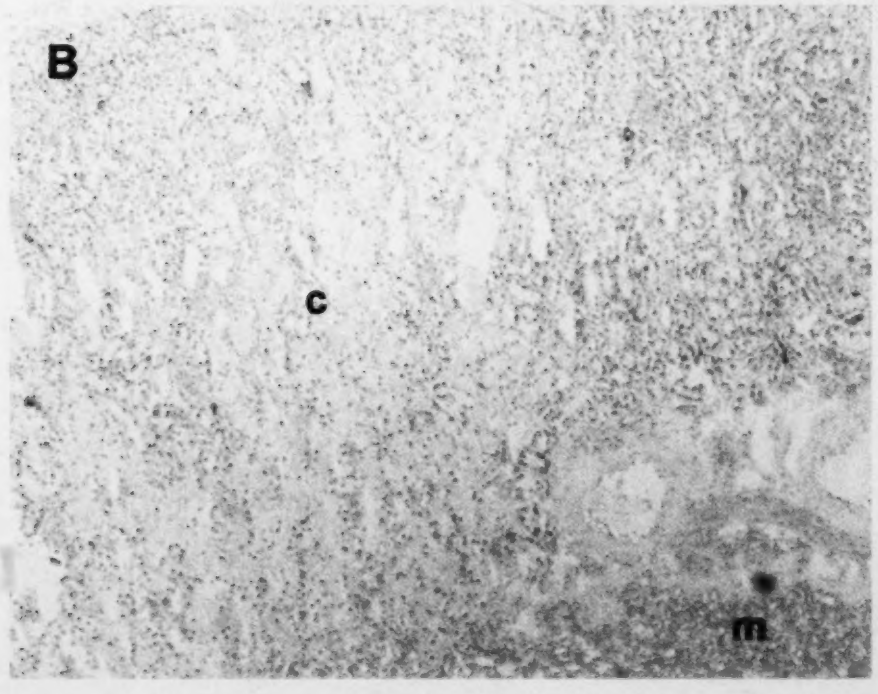
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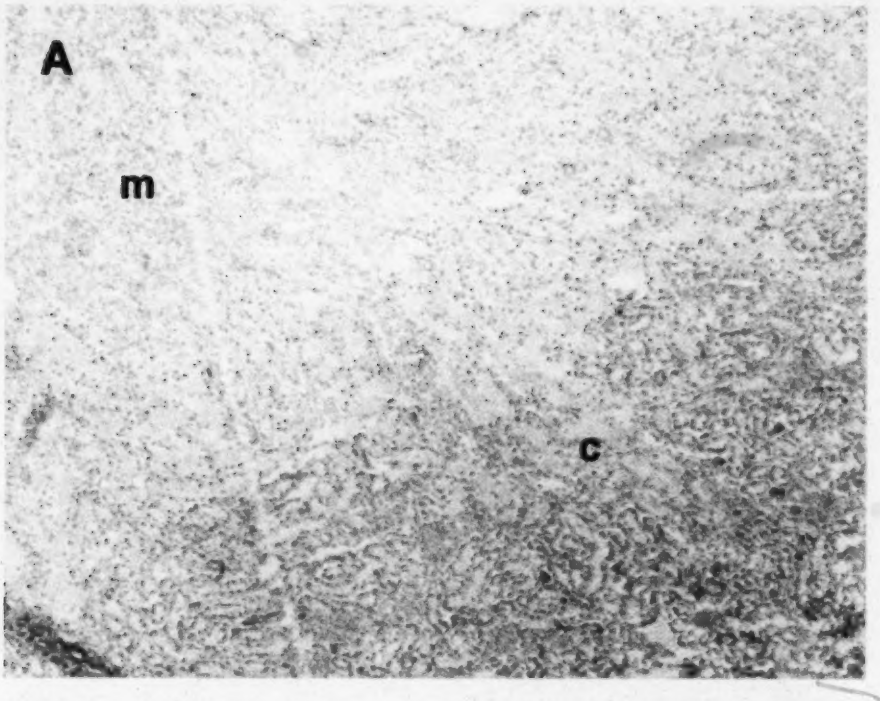
Figure 53. Photomicrographs of active TGF-β1 staining in kidneys from mice administered CsA (d 1 and d 7 post-CsA). BALB/c mice were administered 100 mg/kg CsA daily for 7 d. Frozen sections from CsA treated mice were stained with anti-TGFβ1 affinity purified chicken polyclonal antibody. Kidneys were harvested at d 1 (panel A) or d 7 (panel B). The medulla (m) and cortex (c) are indicated. Both panels are the same magnification (original 100X). These slides are representative of sections from three mice.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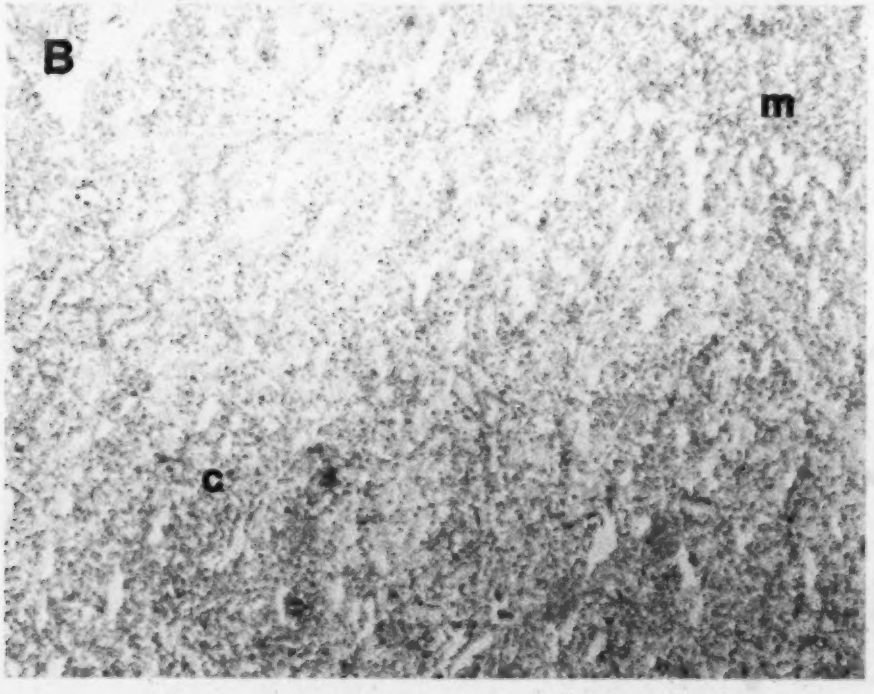
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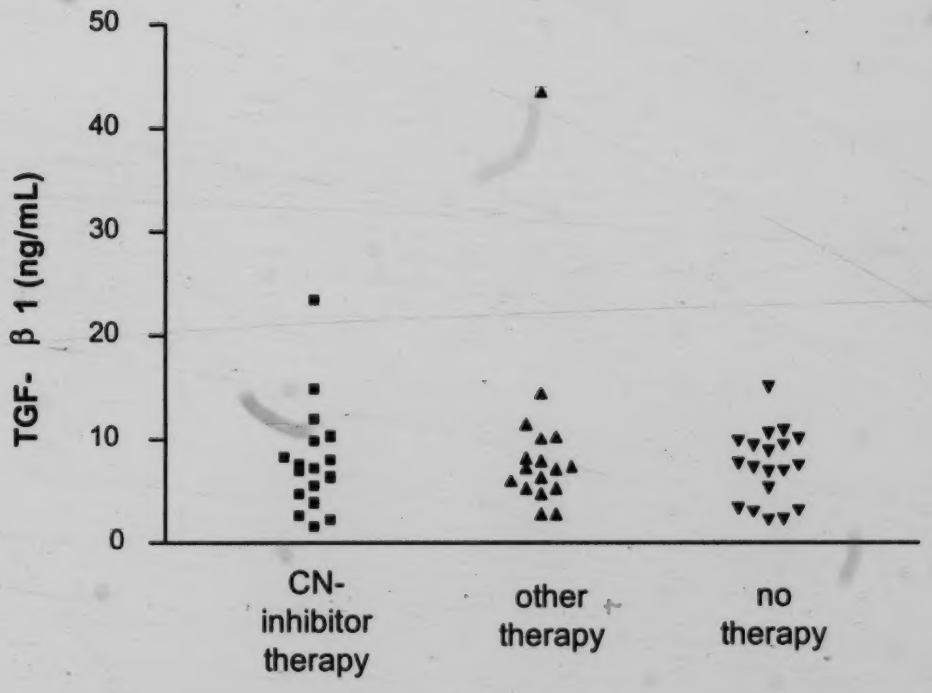

Figure 54. Determination of total TGF-β1 in plasma samples. Plasma samples from patients or volunteers were processed from whole blood, acid-activated, and assayed for TGF-β1 by sandwich ELISA. Patients and volunteers were grouped into three main groups (n=17 for each group): those on CN-inhibitor therapy, other (non-CN inhibitor) therapy, and no immunosuppressive therapy.

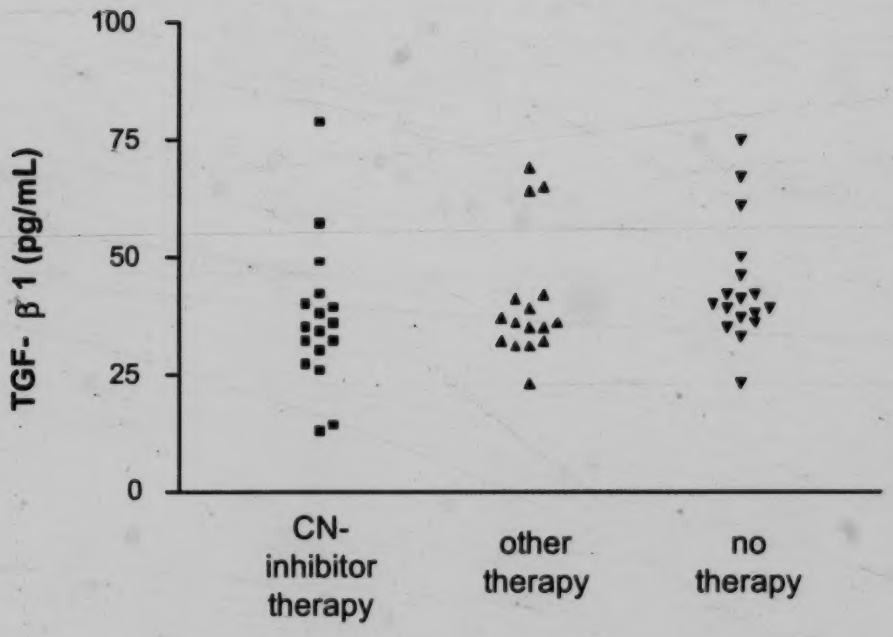

Figure 55. Determination of total TGF-β1 in plasma samples. Plasma samples from patients or volunteers were processed from whole blood and assayed for TGF-β1 by sandwich ELISA. Patients and volunteers were grouped into three main groups (n=17 for each group): those on CN-inhibitor therapy, other (non-CN inhibitor) therapy, and no immunosuppressive therapy.

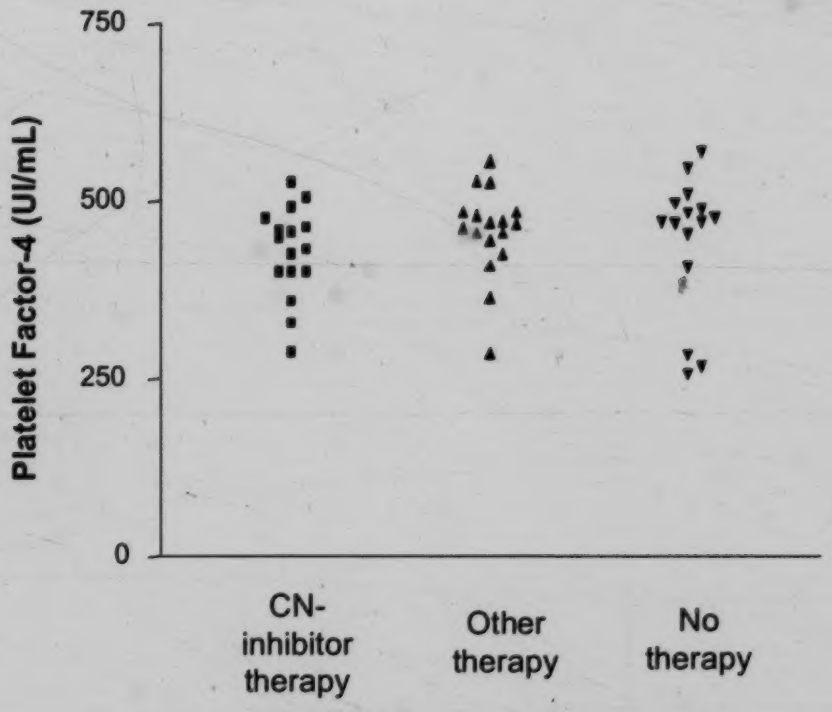

Figure 56. Determination of platelet factor-4 in plasma samples. Plasma samples from patients or volunteers were processed from whole blood and assayed for platelet factor-4 by sandwich ELISA. Patients and volunteers were grouped into three main groups (n=17 for each group): those on CN-inhibitor therapy, other (non-CN inhibitor) therapy, and no immunosuppressive therapy.

Table 5. Comparison of TGFβ1 levels in transplant patients on CN-inhibitor or nonCN-inhibitor therapy with the normal population.

	total TGF-β1 (ng/mL)			active TGF-β1 (pg/mL)		
	mean	median	sd[b]	mean	median	sd[b]
CN inhibitor therapy[a]	7.8	7.1	5.3	37	35	15
other therapy[a]	9.4	7.2	9.3	49	36	40
no therapy[a]	7.3	7.6	3.5	44	40	13
		P=0.9522[c]			P=0.1368[c]	

[a] denotes n=17
[b] denotes standard deviation
[c] denotes Kruskal-Wallis test

Chapter Six

Discussion

I. Summary

Although CsA and FK have been used clinically for the past two decades, questions remain about their mechanism(s) of action. What is the molecular basis for the quantitative differences between CsA and FK? Why are these drugs as effective and specific as they are when their target is a ubiquitously expressed enzyme? What determines the tissue-specific effects of the drugs? The objective of this thesis was to provide a better understanding of the mechanisms and the tissue-specific effects of CsA and FK *in vivo*.

To determine if the quantitative differences between CsA and FK could be explained by their differential inhibition of CN, I first compared the relative activities of FK versus CsA as CN inhibitors and as inhibitors of immunologically-relevant events downstream, namely NFATC2 dephosphorylation and IFN-γ secretion. The FK IC50s for inhibition of CN, NFATC2 dephosphorylation, and IFN-γ production by human PBL were about 25- to 87-fold lower than those of CsA. I also examined CN inhibition by FK *in vitro* in human whole blood and found the IC50 ~20-fold lower than that of CsA. CN activity was also inhibited in humans and in mice administered FK at doses ~30-fold less than CsA.

These studies led to the surprising observation that although FK and CsA completely inhibited NFATC2 dephosphorylation and IFN-γ

secretion, neither drug completely inhibited CN phosphatase activity. Moreover, at saturation, FK inhibited less CN phosphatase activity than CsA despite its greater molar potency for *in vivo* immunosuppression and *in vitro* inhibition of cytokine production. Various technical explanations for the incomplete inhibition, such as substrate dependence, were ruled out. The incomplete inhibition was not cell- or tissue-specific, although the degree of incomplete inhibition varied among tissues. I then found that the incomplete inhibition could be overcome by the addition of the appropriate immunophilin to the assay buffer, implying that active immunophilins could be limiting for inhibition of CN phosphatase activity by CsA and FK. It also suggested that active FKBPs were more limiting than active CyPs for CN inhibition. Indeed, I subsequently showed that protein expression of FKBP12 was generally lower than that of CyPA in mouse tissues. Further examination revealed that the limitation was not a result of physical loss during the process of extraction and that active immunophilins were limiting only at high drug concentrations.

Since the active immunophilin concentration could limit the degree of CN inhibition at high drug concentrations, I hypothesised that active immunophilins may differentially limit the effect of FK and CsA on CN in different tissues. Comparing the maximum degree of CN inhibition by FK in different tissues, I found that it ranged from 32%

(brain) to 60% (liver) and was generally lower than that by CsA. In addition, the maximum inhibition could be increased to near completion with exogenous FKBP12. Active immunophilins were also limiting only at high drug concentrations in these tissues. These experiments led to the unexpected observation that the *in vitro* IC50s for FK in tissue homogenates were in the same range as those for CsA. The discrepancy between these and earlier results was due to cell disruption. I found that the IC50 for CN inhibition was greater for FK if drug exposure occurred after cell disruption. That is, the IC50 for FK was 10-fold lower if intact cells were treated rather than cell lysates. Interestingly, the IC50s for CsA were the same regardless of whether intact or disrupted cells were treated.

Finally, to determine whether induction of TGF-β1 was a mechanism through which CsA acted, I examined the relationship between CsA treatment and TGF-β1 *in vitro* and *in vivo*. While spleen cell suspensions could not be induced to secrete TGF-β1 under any of the conditions tested, PBL suspensions could be induced to secrete TGF-β1 following stimulation with PMA. CsA treatment of PBL did not induce TGF-β1 secretion nor did it affect the induction of TGF-β1 secretion by PMA. TGF-β1 mRNA was also not altered in kidneys of mice fed high doses of CsA daily for 7 days. Immunohistochemical analysis of these kidneys also showed no changes in latent or active

TGF-β1 protein expression following CsA treatment. Finally, a population study of transplant patients revealed no differences in plasma TGF-β1 levels between those on CN-inhibitor therapy and those on other immunosuppressive therapy. Moreover, no differences were observed between transplant patients and the normal population.

A possible basis for the quantitative differences between CsA and FK, the implications of incomplete CN inhibition by CsA and FK, and the study of the relationship between CsA and TGF-β1 will be discussed in detail.

II. The molecular basis for the greater molar potency of FK vs. CsA.

The present data support the model that differential inhibition of CN is a molecular basis for the quantitative differences between CsA and FK. The differences in IC50s (for inhibition of CN, NFATC2 dephosphorylation, IFN-γ secretion) between FK and CsA in human PBL and whole blood correlate with the ratio of the therapeutic doses and blood concentrations of the two drugs, and with their *in vitro* effects on immune activation. CN phosphatase activity is also inhibited in patients on FK-based immunosuppressive therapy. At 1 h and 12 h post-FK, CN was inhibited 30-40% in whole blood. This is the first direct demonstration of CN inhibition in patients following FK

administration. This laboratory has previously demonstrated CN inhibition between 30-70% in transplant patients on CsA-based therapy (85). At the moment, comparisons between FK and CsA are difficult as more time points for analysis as well as more patients on FK-based therapy are required. However, I have shown that FK suppresses CN activity in transplant patients at ~20 to 40-fold lower concentrations than CsA and that the suppression of CN correlates with the clinically observed immune suppression. Together, these *in vitro* and *in vivo* data support the model that the mechanism of immune suppression of both CsA and FK is via CN inhibition and that differential inhibition of CN activity is one major reason for the quantitative differences between these two drugs.

Similar to what has been shown by this and other laboratories in patients on CsA therapy (83;84;263;264;278-280), I have demonstrated that CN activity is only partially inhibited in transplant patients on FK therapy. The CN inhibition seen at these clinical levels is compatible with the *in vitro* IC50 for FK in human whole blood (12.7 ng/mL, 95% CI 8.9 – 18.1 ng/mL). This partial inhibition of CN activity is also consistent with an earlier report that IL-2 production was suppressed and nuclear translocation of NFATC was inhibited in patients treated with FK (281). The measurement of CN activity in conjunction with determination of FK drug levels may help physicians

to better optimize the dosing regimen on an individual patient basis. When first introduced clinically, many of the observed toxicities associated with FK were a result of improper dosing. Since drug metabolism among individuals and the sensitivity of individuals to FK may differ, a direct measurement of the drug's biologic activity is attractive. Many FK assays detect not only the parent drug but also FK metabolites. The effect of FK metabolites on the inhibition of CN activity by FK is not known. In addition as generic formulations of CsA become available, the ability to measure CN activity may become useful. These formulations are not likely to be equivalent or readily interchangeable with CsA. Monitoring CN inhibition in patients treated with these new formulations might help to clarify quickly issues of dosing. Although the value of monitoring CN activity in individual patients still needs to be established, the concept is appealing.

Comparing CN inhibition by CsA and FK in nonimmune tissues, I found that the IC50s were in the same range as those previously determined for CsA. The discrepancy between these results and the ones in human PBL and whole blood could be explained by cellular disruption. The IC50 of FK was lower if intact cells vs. cell lysates were treated. This suggested that some special condition existed in intact cells and was no longer present following cell disruption. Interestingly, the IC50s for CsA were the same regardless of whether

intact or disrupted cells were treated, implying that the condition was not as important for CsA. Compartmentalisation of FKBP but not CyP with CN is one possible explanation. This explanation would be consistent with the observation that FKBPs are more limiting than CyPs for CN inhibition by FK and CsA, respectively, in the assay. The protein expression of FKBP12 in tissues was also generally lower than that of CyPA. Compartmentalisation of FKBP would allow for more specific targeting of FK to CN and thus potentially contribute to its higher molar potency. There may however be other possible explanations for these observations. For example, FK may concentrate in cells to a greater extent than CsA. Regardless of the basis, these observations highlight potentially important mechanistic differences between CsA and FK.

III. The implications of incomplete CN inhibition and limiting immunophilins

Contrary to previous assumptions, these data generated in this sutdy suggest that immunophilins may be limiting for inhibition of CN phosphatase activity by FK and CsA at high drug concentrations. Many distinct isoforms of CyP and FKBP exist, both as free species and anchored to membranes (282)(283). As discussed in chapter one, not all immunophilins form active drug:immunophilin complexes that

bind and/or inhibit CN. Thus, although immunophilins have been described in literature as ubiquitous and abundant proteins, those which can form drug:immunophilin complexes that go on to inhibit CN phosphatase activity may be less abundant and thereby potentially limiting for CN inhibition.

The observation that active immunophilins are limiting for complete inhibition of CN phosphatase activity but not for complete inhibition of IFN-γ production or NFATC2 dephosphorylation raises the possibility that a compartmentalized or spatially restricted subset of CN is critical for immune function. Scaffolding, anchoring, and adapter proteins give structure to phosphatase and kinase signaling pathways by regulating protein:protein interactions (284). These proteins contribute to the specificity of a signal by recruiting enzymes into signaling networks or by placing enzymes close to their substrates. They regulate when and where protein kinases and phosphatases are activated in the cell. For example, receptors for activated C kinase (RACKs) tether PKC to the cytoskeleton or at submembrane sites through protein-phospholipid interactions (285). As a ubiquitous enzyme that plays a role in a number of cellular processes, CN also participates in a number of protein-protein interactions with various membrane bound proteins such as A-kinase anchoring protein (AKAP) (118), bcl-2 (123), and the IP_3 receptor (IP_3R) via FKBP12 (138). CN

has been shown to be associated with the cytoskeleton (286) as well as the nucleus (287;288). CN also has membrane-binding potential that could affect its localization (126;289;290). Thus CN is probably not a homogenous population and specific protein-protein interactions may differentially regulate the different subsets of CN. The key downstream events in the immune response may therefore be affected by inhibition of only a subset of immunologically-relevant CN. This selectivity could be due to compartmentalization of CN functions, with differing availability of immunophilins among the compartments.

These observations must be interpreted in light of differences between the assay for CN phosphatase activity and, for example, the assays for IFN-γ production and NFATC2 dephosphorylation. CN phosphatase activity must be assessed in disrupted cells. NFATC2 dephosphorylation and IFN-γ production reflect conditions in intact cells, and thus not subject to experimental manipulation such as cell lysis. By trying to assess in disrupted cells what occurs in intact cells, we may be creating conditions in the assay which do not occur in the cell. For example, immunophilins or other factors important for inhibition may be differentially lost relative to CN. Moreover, CN can be inhibited by proteins other than immunophilins plus drug, such as cabin 1/cain and CHP (125;126;129). Cell lysis might disrupt such inhibitory interactions and release CN from being inhibited in the cell to

being active in the assay. Although these observations may be artifacts of the CN assay, the question of why CsA and FK perform differently in the assay remains unaswered or unresolved. It suggests that there may be an important and previously unknown difference in mechanism of action between the two drugs.

The abundance of active immunophilins differentially limits the effect of FK and CsA on CN in different tissues, cell types, or subcellular compartments. Some tissues may be protected by low levels of active immunophilin while others may be more susceptible because of their high levels. The tissue-specific effects of FK and CsA may be related to the differential levels of active immunophilins in the tissues. Mouse bone marrow-derived progenitor mast cells, for example, were shown to be sensitive to inhibition by CsA but resistant to FK; the resistance was associated with a deficiency in FKBP12 and over-expression of FKBP12 in these cells resulted in sensitisation of these cells to FK (291;292). The relative ratio of CN to active immunophilins as well as the ratio of active to inactive immunophilins may be important in determining the effect of CsA or FK on a particular tissue. For example, CsA causes nephrotoxicity and hepatotoxicity but not cardiotoxicity. At high CsA concentrations, the extent of CN inhibition in the kidney and liver is greater than in the heart. I have shown that inhibition of CN activity by CsA in the heart can be

increased with the addition of CyPA. A greater abundance of active immunophilin (relative to CN) in the kidney and liver than in the heart may account for this difference. Ten-fold more CyP has been previously demonstrated in kidney and in liver than in heart (293). Our laboratory has also demonstrated higher levels of both CyPA and CyPB in kidney and liver vs. heart (Kung, L. *et al* submitted). Thus a higher ratio of active immunophilin to CN in the kidney and liver (compared to heart) correlates with a greater degree of CN inhibition by high concentrations of CsA in these tissues. Tissues, such as the heart, with lower ratios of active immunophilin to CN may be more protected from the peak effects of high CsA concentrations. Thus varying levels of active immunophilins among different tissues are a likely basis for the different degrees of CN inhibition among these tissues at saturating CsA concentrations (262). The level of active immunophilins also appears to correlate with the sensitivity of the tissue to high concentrations of CsA. Inactive immunophilins may be important because they may reduce the amount of drug available for the active immunophilins at non-limiting drug concentrations or act as reservoirs at high drug concentrations.

The ability of the drug to accumulate in the tissue as well as the maximum CN inhibition possible in that tissue may contribute to the susceptibility of that organ to toxicity. This laboratory has shown that

CsA accumulates more in the kidney and the liver than in the heart. FK enters most tissues and differentially inhibits CN activity in those tissues. Similar to CsA, FK accumulated more in kidney than in spleen or whole blood and this is consistent with its risk of nephrotoxicity. The only exception to this model is the brain. CN in the brain is resistant to CN inhibition and the brain accumulates comparatively low levels of CsA and FK. What then is the mechanism of neurotoxicity? It is possible that it is the inhibition of the isomerase activity of the CyP by CsA and not the inhibition of CN by CsA:CyP that disrupts brain function. Bennett *et al* showed significant inhibition of memory formation by CsA analogs that bound CyP but did not inhibit CN. However, the study was done in chicks and the drugs were injected intracranially (294). It remains to be determined what drug concentrations are required in the mouse and human for such effects and whether these concentrations are achieved following oral or i.v. administration of CsA.

A number of observations suggest that inhibition of CNAα in the brain as the basis for neurotoxicity. Foremost, CNAα-deficient mice have deficits in learning and memory (152). Furthermore, CN regulates neurotransmitter release (140;295;296), increases stability of microtubules in the brain (139;156), regulates production of nitric oxide in the brain (74), and potentiates long-term depression (141).

Interestingly, active CyPs appear more limiting than active FKBPs in the brain (i.e. greater maximum CN inhibition by FK than CsA). This correlates with the greater risk of neurotoxicity with FK than with CsA. The average CN inhibition in the brain may not be the pattern for particular cells or intracellular compartments; that is, CsA may accumulate and inhibit CN in specific areas of the brain. Indeed, different regions of the brain have been shown to respond differently to CsA (266). CN, CyP, and FKBPs are found in assemblies with various membrane bound proteins such as TGF-βR, A-kinase anchoring protein, bcl-2, and the IP_3R (118;123;138;282;297;298). Compartmentalization of CN functions, differing availability of immunophilins, and targeting of CsA within the cell could also result in selective inhibition of CN subsets.

It is not certain whether minute amounts of CsA enter the brain, falling below the level of detection for the assay, or whether CsA does not enter at all. FK has been shown to peak at 1 h post-dose in the brains of mice and to slowly decrease over time (299). P-glycoprotein plays an important role in the efflux of FK from the brain as its absence results in rapid and sustained accumulation of FK in the brain. The expression of P-glycoprotein in humans is less characterized. If it is lower in humans than mice, then more CsA and FK would enter the brain than what is seen in mice and more CN

inhibition may be occurring than presently thought. Alternatively, CsA and FK may influence the microcirculation in the brain, affecting CNS function without actually entering the brain (300;301). Nevertheless, with the exception of the brain, these data are compatible with the hypothesis that the toxicity and efficacy of CsA and FK are both due to CN inhibition. CN directly and indirectly modulates the activity of a diverse range of intracellular proteins such as NFATC, NFκB, nitric oxide synthase, elk-1, IP_3-R, and various neuroproteins. The inhibition of CN activity against this broad range of substrates is a possible biochemical basis for the effects of CsA.

IV. Investigating the relationship between TGF-β1 and the CN inhibitors.

Transforming growth factor-beta (TGF-β) has been implicated in a various phenomena associated with CsA and to some extent FK. These include the immunosuppressive action of CsA, the nephrotoxicity of CsA, and, recently, the tumor progression associated with CsA (302-307). But many of these observations are controversial. The study of TGF-β1 is difficult because of its complex regulation at the transcriptional, translational, and post-translational levels. Its ubiquity and multi-functionality also hinder efforts to interpret clinical and experimental findings. TGF-β1 has been associated not only with

diseases (e.g. certain cancers) but also with normal physiologic processes (e.g. wound healing).

The first study reporting a link between CsA and TGF-β1 is not conclusive (308). Li *et al* examined TGF-β1 expression at the mRNA level by competitive PCR and reported a two-fold increase following pharmacologic stimulation and CsA (100 ng/mL) treatment of T cells *in vitro*. Interestingly, an increase was observed at 1 h but not 4 h, 16 h, or 40 h. This paper also examined TGF-β1 protein in cell culture supernatants and showed that neutralizing anti-TGF-β1 antibody suppressed the bioactivity of TGF-β1 in cell culture supernatants. The bioactivity of TGF-β1 in cell culture supernatants was measured by its inhibition of growth of mink lung epithelial cells. Although not specifically stated, the culture medium must have contained some serum to support cell culture for 40 h. A negative control of the culture media alone or even the supernatant of untreated T cells was not included for comparison to supernatants from CsA-treated T cells. Various sources of serum are known to contain significant amounts of TGF-β. The TGF-β bioactivity detected in their biologic assay could simply have been from the serum in the culture media and may not necessarily produced by the T cells following CsA treatment.

Some studies have demonstrated increased expression of TGF-β1 in mice and patients following administration of CsA (268;309;310).

Elevated TGF-β1 has been correlated with hypertension (311) and fibrosis (303;306) associated with FK and CsA treatment. However, *in vivo* studies have also been limited by experimental technique and design. The measurement of circulating TGF-β1 protein is especially debated. The detection system, sample dilution, measurement of plasma vs. sera, and sample handling are all variables which affect the results obtained and subsequently the conclusions made. One study linking the immunosuppressive and nephrotoxic effects of CsA with TGF-β1 in mice, for example, used the Promega detection system for TGF-β1 and examined sera diluted 1/150 (310). First, the Promega kit was rated the lowest in the survey of TGF-β1 detection systems. Second, the assessment of TGF-β1 in serum is discouraged because of the release of TGF-β1 during the clotting process. Finally, large dilutions of the sample are also discouraged because of dilutional nonlinearity (270).

As outlined in chapter 5, I have endeavored to minimize the analytical problems by using standardized and robust methods for sample handling and well-validated techniques for measurement. Immunohistochemistry is without a doubt the weakest method of analysis. This method is limited by the availability of reliable reagents (namely antibodies) and suitable controls (namely the TGF-β1 knockout mouse). I chose antibodies that were previously shown to

successfully detect and distinguish between the latent and active forms of TGF-β1 in mouse mammary tissue (247;273;274). I have also optimized the system to minimize nonspecific background staining and have provided the appropriate controls. These data are at least as believable as those already published. Conclusions based on immunohistochemical staining are also limited by the sensitivity of this method. Cisplatin treatment induces intense renal injury. The effects of CsA may be more mild and these changes may not be detectable by immunohistochemistry. However, the conclusions based on immunohistochemistry are supported by those based on the Rnase protection assay and Taqman.

In vivo studies are also not easily interpretable because a number of processes are occurring simultaneously and an observed effect cannot be attributed solely to one process. Many non-specific conditions may alter TGF-β levels in a particular experiment. Because TGF-β has numerous activities in biologic systems, it is subject to complex control and can be postulated to have almost any beneficial or detriment effect. As a ubiquitous factor in growth, healing, and fibrosis, TGF-β mRNA or product levels are altered in a great many processes. Thus the potential for its involvement is considerable, but the chances for finding spurious associations between TGF-β and disease processes are high. TGF-β is found in most processes where there is

tissue injury, inflammation, or scarring and its role in these processes is likely to be complex. In situations where an association might be found, it is difficult to conclude a cause-effect relationship (solving the "which came first—the chicken or the egg" dilemma). For example, did TGF-β cause the fibrosis or did the fibrosis result in the detection of TGF-β?

A cause-effect relationship between CsA and oncogenesis has also been pursued. CsA in mice increases progression of transplanted cancer cell lines that are regulated by TGFβ. TGF-β-dependent human lung adenocarcinoma cells that are non-invasive *in vitro* can be induced to an invasive phenotype with CsA treatment. The phenotypic changes were reversible and could be inhibited using anti-TGF-β antibodies. Metastic growth of tumor cells were induced *in vivo* in SCID-beige mice, which also suggested that the tumor-progressive effect of CsA was on the cancer cells directly and not attributable to suppression of the host immune system (312). However, this effect of CsA has only been demonstrated in this artificial cancer model and cannot be extrapolated to oncogenesis but rather to growth of TGF-β-dependent tumor cell lines. No similar effect of CsA has been shown on precancerous cells and CsA has not been implicated in the conversion of benign tumors to cancerous tumors. Moreover, CsA in human populations has not been shown to affect the incidence or

progression of tumors any more than any other immunosuppressive. Thus there is no evidence to date that the CN inhibitors alter cancer risks by TGF-β mechanisms or any other mechanism other than by immunosuppression.

V. Future directions

The data presented here contribute to a better understanding of the molecular basis for the clinical effects of the current CN inhibitors and have implications for the development of new agents. Intracellular tracking experiments could be done to address the question of whether FK but not CsA target to certain sites in the cell, as suggested by these data. New drugs could be developed which target CN relevant for immune function while leaving general CN activity intact. CN directly and indirectly modulates the activity of a diverse range of intracellular proteins such as NFATC, NFκB, nitric oxide synthase, elk-1, IP_3-R, and various neuroproteins. The inhibition of CN activity against this broad range of substrates is a possible biochemical basis for the effects of CsA and FK.

For more specific CN inhibition, a finer dissection and better understanding of the signaling pathways in which CN is involved is imperative. More knowledge of the different drug binding proteins in the tissues may also be helpful in designing more specific CN

inhibitors. For example, if a certain binding protein was found to be preferentially expressed in immune cells, analogs of CsA or FK might be engineered to target it. The overall aim would be to preferentially target the relevant subset of CN and spare CN pathways that if inhibited would lead to toxicity. These therapies could achieve greater specificity, less toxicity, and thus greater drug efficacy.

VI. Conclusions

1. Differential CN inhibition by CsA and FK is one possible molecular basis for the difference in molar potency.

2. Active immunophilins can be limiting for inhibition of CN phosphatase activity at high drug concentrations.

3. Complete inhibition of IFN-γ secretion and NFATC dephosphorylation and incomplete inhibition of CN phosphatase activity suggest that there is an immunologically relevant subset of CN.

4. The maximum CN inhibition by FK is generally lower than that by CsA and suggests that FKBPs are more limiting than CyPs. Protein levels of FKBP12 are generally lower than that of CyPA.

5. Active immunophilins are differentially limiting among tissues and low levels might protect some tissues at high drug concentrations while other tissues may be more susceptible because of high levels.

6. The molar potency of FK is greater in intact cells than in cell extracts while the molar potency of CsA is unaffected by cellular disruption. Compartmentalisation of CN with FKBP but not CyP in intact cells may also contribute to the greater molar potency of FK.

7. CsA does not alter the expression of TGF-β1 by mouse or human immune cells at the mRNA or protein level *in vitro*, implying that induction of TGF-β1 in immune cells is not a mechanism through which CsA acts.

8. CsA treatment of mice does not affect TGF-β1 mRNA or protein in the kidney, indicating that upregulation of TGF-β1 is not a therapeutic or nephrotoxic mechanism through which CsA operates *in vivo*.

9. Plasma levels of total and active TGF-β1 are not different between transplant patients on CN-inhibitor or non-CN inhibitor based therapy and the normal population. These results support the

argument that augmentation of TGF-β1 is not a mechanism through which the CN inhibitors act.

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Education

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Honors and Awards

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Faculty of Medicine 75th Anniversary Award, University of Alberta, 1995/6

Dean's List, University of Alberta, 1990/1-94/5

Canada Scholarship, University of Alberta, 1990/1-94/5

Harry W. Bass Memorial Bursary, University of Alberta, 1990

Rutherford Scholarship, Province of Alberta, 1987/88-1989/90

City of Edmonton Honors Award, 1990

Publications

1. **Kung, L.**, Batiuk, T.D., Noujaim, J., Paolomo, S., Helms, L.M., and Halloran, P.F. Tissue distribution of calcineurin and its sensitivity to inhibition by cyclosporine. (accepted to American Journal of Transplantation)

2. **Kung, L.** and Halloran, P.F. Calcineurin-targetted inhibition of the immune response. Therapeutic Immunosuppression (Editor: Thomson, A.W.), Klurow Publishers (in press)

3. **Kung, L.** and Halloran, P.F. Immunophilins may limit calcineurin inhibition by cyclosporine and tacrolimus at high drug concentrations. Transplantation 70(2):327-35, 2000.

4. **Kung, L.**, Gourishankar, S., and Halloran, P.F. Molecular pharmacology of immunosuppressive agents in relation to their clinical use. (Review) Current Opinion in Organ Transplantation 5:268-275, 2000.

5. Halloran, P.F., Helms, L.M., **Kung, L.**, and Noujaim, J. The temporal profile of calcineurin inhibition by cyclosporine *in vivo*. Transplantation 60:1356-1361, 1999.

6. Halloran, P.F., Noujaim, J., and **Kung, L.** Calcineurin and the biological effect of cyclosporine and tacrolimus. Transpl Proc 30: 2167-2170, 1998.

7. Batiuk, T.D., **Kung, L.**, and Halloran, P.F. Evidence that calcineurin is rate-limiting for primary human lymphocyte activation. Journal of Clinical Investigation 100:1894-1901, 1997.

Manuscripts in preparation:

1. **Kung, L.,** Loertscher, R., Melk, A., Sawitzki, B., Ramassar, V., and Halloran, P.F. Cyclosporine does not directly affect TGF-β1 expression *in vivo* or *in vitro*.

2. **Kung, L.**, Noujaim, J., Helms, L.M., Malcolm, H., and Halloran, P.F. The measurement of calcineurin phosphatase activity in human and mouse.

Presentations at International Meetings

1. **Kung, L.**, Sedlemeyer, A., and Halloran, P.F. The higher molar potency of tacrolimus may reflect pre-assembly of the binding protein with calcineurin. (poster presentation) American Society of Transplantation, 2001.

2. **Kung, L.**, Loertcher, R., and Halloran, P.F. Cyclosporine and the TGF-β secretion by mouse and human immune cells. (poster presentation) Canadian Society of Transplantation, 2001.

3. **Kung, L.** and Halloran, P.F. A new explanation for the greater potency of tacrolimus compared to cyclosporine. (poster presentation) American Association of Immunologists, 2001.

4. **Kung, L.** and Halloran, P.F. Cyclosporine and tacrolimus: complete inhibition of calcineurin immune function with incomplete inhibition of calcineurin phosphatase activity. (oral presentation) American Society of Transplantation, 1999

5. **Kung, L.**, Noujaim, J., and Halloran, P.F. Immunophilin concentrations can be limiting for calcineurin inhibition by tacrolimus and cyclosporine. (poster presentation) American Association of Immunologists, 1999

6. **Kung, L.**, Noujaim, J., and Halloran, P.F. Tacrolimus (FK) and Cyclosporine (CsA): Different effects on calcineurin (CN). (oral and poster presentation) Canadian Society for Immunology, 1999.

7. **Kung, L.** and Halloran, P.F. Mechanism of action of cyclosporine a (CsA) and tacrolimus (FK) on calcineurin. (poster presentation) American Society of Transplant Physicians, 1998.

8. **Kung, L.** and Halloran, P.F. Tacrolimus (FK) and cyclosporine (CsA): A different mechanism of inhibition on calcineurin (CN) (poster presentation) American Association of Immunologists, 1998

9. **Kung, L.** and Halloran, P.F. Neither FK nor CsA completely inhibit CN phosphatase activity against peptide substrates in vitro or in vivo. (poster presentation) World Congress of the Transplantation Society, 1998.

10. **Kung, L.** and Halloran, P.F. The mechanism of action of cyclosporine A and tacrolimus. (poster presentation) Canadian Society for Immunology, 1997

11. **Kung, L.** and Halloran, P.F. Differences between tacrolimus and cyclosporine in action on calcineurin. (poster and mini-symposium) American Society for Nephrology, 1997

TEACHING AND LEADERSHIP

Lectures and Presentations

University of Alberta Immunology Network Student Retreat
June 2000

Department of Medical Microbiology and Immunology
May 2000
Science and Peanut Butter Seminar

Alberta Nephrology Days
April 1999

University of Alberta Immunology Network Retreat
September 1997

Teaching and Employment

Department of Medical Laboratory Science, University of Alberta
Instructor for Medical Laboratory Science (MLS) 475,
1998 and 1999

Department of Medical Microbiology and Immunology, University of Alberta
Teaching Assistant for Interdepartmental (INTD) 452, 1998

University of Alberta Teaching Symposium
Participant, 1996 and 1997

Leadership

Department of Medical Microbiology and Immunology, University of Alberta
Volunteer cohort, 1996-present

University of Alberta Immunology Network Retreat
Registration and Fees, 2000

University of Alberta Immunology Network
Studentship committee, 1999-2000

University of Alberta Immunology Network
Distinguished Immunologist Seminar Series Coordinator, 1997-2000

University of Alberta Immunology Network
Student council member/representative, 1997-2000

University of Alberta Immunology Network Retreat
Public Relations, 1999

University of Alberta Election Procedures Committee
Member, 1999

University of Alberta Immunology Network Retreat
Food committee, 1998

Faculty of Graduate Studies and Research Council
Graduate student representative, 1997/98

Academic Appeals Board
Graduate student alternate member, 1997/98

Graduate Student Association, University of Alberta
Department of Medical Microbiology and Immunology Student Representative, 1995-1996